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This doctoral thesis thus focuses on an evolutionary genotype-phenotype study in mammals, examining sequence and regulatory evolution of protamines and accompanying changes in sperm head phenotype. Analyses were undertaken of protamine gene and regulatory sequence evolution as well as comparative gene expression aiming to show the importance of protamine function on sperm head size and shape. Species exhibiting different levels of sperm competition served as models to assess relationships between sperm competition and sperm head phenotype brought about by selective pressures acting on these proteins. The results presented here are important for a better understanding of the complex interplay between selective forces potentially acting on DNA sequences and cell phenotype. Gene and regulatory sequences were obtained by standard PCR techniques as well as gene walking PCR. Quantitative PCR was used to obtain gene expression data. Genotype-phenotype association studies were developed using root-to-tip dN/dS (nonsynonymous / synonymous substitutions rate ratio) to account for evolutionary rates and phylogenetic generalized least squares analyses to compare genetic and morphometric data.

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PROTAMINES – SELECTION AND FUNCTION IN MAMMALS

Lena Lüke – 2015

Universidad Autónoma de Madrid
Departamento de Biología Molecular

PROTAMINES

SELECTION AND FUNCTION IN MAMMALS

LENA LÜKE

Tesis Doctoral
Museo Nacional de Ciencias Naturales (CSIC)

Universidad de Autónoma de Madrid

Faculdad de Ciencias

Departamento de Biología Molecular

PROTAMINES – SELECTION AND FUNCTION IN MAMMALS

Memoria presentada por Lena Lüke para optar al grado de doctora en Ciencias Biológicas

Dirigida por Eduardo Roldan Schuth, Profesor de Investigación de Consejo Superior de Investigaciónes Cientificas (Museo Nacional de Ciencias Naturales) y Montserrat Gomendio Kindelan.

Tutelada por Lourdes Ruíz Desviat, Profesora Titular del Departamento de Biología Molecular de la Universidad Autónoma de Madrid.

El Doctorado

Lena Lüke

VºBº del Director

Eduardo Roldan Schuth

VºBº del Co-director

Montserrat Gomendio Kindelan

VºBº del Tutor

Lourdes Ruíz Desviat

Madrid, Junio de 2015

to my son Dante and my daughter Matilda,
be who you want to be.

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Fotos on title page are taken from http://www.arkive.org/ and http://www.carpathianbasinspecies.eu/ (22. May 2015)

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Después de todo tu eres la única muralla,
si no te saltas nunca darás un solo paso

Luis Alberto Spinetta

CONTENTS

ABBREVIATIONS

OXPHOS	OXidative PHOSphorylation
SEMG	SEMenoGelin
SVS	Seminal Vesicle Secretory protein
ADAM	A Disintegrin And Metalloproteinase
SPAM	SPerm Adhesion Molecule
DNA	DeoxyriboNucleic Acid
Prm	Protamine gene
PRM	Protamine
pre-PRM2	Protamine 2 precursor
cleaved PRM2	Protamine 2 domain which is cleaved off during processing
mature PRM2	Protamine 2 domain remaining after complete processing
cleaved *Prm*2	part of Protamine 2 gene sequence coding for cleaved PRM2
mature *Prm*2	part of Protamine 2 gene sequence coding for mature PRM2
MAR	nuclear Matrix Attachment Regions
N-terminal region	Amino terminal region of amino acid sequence
C-terminal region	Carboxy terminal region of amino acid sequence
MYA	Million Years Ago
mRNA	messenger RiboNucleic Acid
HL	Sperm head length
HW	Sperm head width
HA	Sperm head area
PGLS	Phylogenetic generalized least squares

CHAPTER 1
RESUMEN – SUMMARY

Los protaminas son proteínas básicas localizadas en el nucleo espermático y tienen un papel crucial en la reorganización y protección del genoma paterno que tiene lugar en las fases finales de la formación de los espermatozoides. Las alteraciones en la expresión génica de las protaminas tienen un impacto importante sobre la forma de la célula espermática, la fertilidad masculina y la supervivencia embrionaria. Existe gran variabilidad en los fenotipos de los espermatozoides de mamíferos. Se piensa que esta diversidad se ha producido principalmente como resultado de la selección sexual postcópula (es decir, la competición espermática y la elección críptica de la hembra). Para comprender cómo se ha desarrollado esta diversidad, es importante entender los patrones selectivos comunes entre especies y aquellos que son diferentes, así como los cambios en los genes que son clave para la formación y función de los espermatozoides. Se ha propuesto que la evolución de los genes de las protaminas se produce bajo la influencia de la competición espermática. Esta tesis doctoral se ha centrado por lo tanto en un estudio evolutivo de relaciones genotipo-fenotipo en mamíferos, examinando la evolución de la secuencia y la regulación de las protaminas y los cambios en el fenotipo de las cabezas de los espermatozoides. Se realizaron análisis de genes de protaminas y la evolución de las secuencias reguladoras, así como de la expresión génica comparada con el fin de mostrar la importancia de la función de las protaminas en el tamaño y la forma de la cabeza espermática. Se examinaron especies que exhiben diferentes niveles de competición espermática como modelos para estudiar las relaciones entre la competición espermática y el fenotipo de la cabeza de los espermatozoides. Los resultados de este estudio son importantes para conocer mejor la compleja interacción entre las fuerzas selectivas que potencialmente pueden actuar sobre las secuencias de ADN y el fenotipo celular. Las secuencias del gen y de los dominios reguladores se obtuvieron mediante técnicas estandar de PCR así como PCR con desplazamiento sobre el gen. Se empleó PCR cuantitativa para obtener información sobre expresión génica. Los estudios de asociación genotipo-fenotipo se desarrollaron calculando el "ratio dN/dS desde base al extremo" (ratio de tasas de substituciones no sinónimas / sinónimas) para conocer las tasas evolutivas, así como análisis filogenéticos generalizados de cuadrados mínimos

(PGLS) para comparar datos genéticos y morfométricos. Los resultados de este estudio han revelado un patrón sorprendentemente complejo de restricción selectiva, así como el efecto de la competición espermática sobre la divergencia de la secuencia de protaminas y de niveles de expresión. Más aún, estos resultados representan una evidencia sólida del efecto de las protaminas sobre la forma y tamaño de la cabeza de los espermatozoides.

Protamines are basic sperm nuclear proteins with a crucial role in the reorganization and protection of the paternal genome that takes place during the final stages of sperm formation. Alterations in protamine gene expression have a major impact on shape of the sperm cell, male fertility and embryo survival. There is great variability in mammalian sperm phenotypes and it is thought that such diversity has been brought about largely by postcopulatory sexual selection (i.e., sperm competition and cryptic female choice). To understand how this diversity is established it is important to examine both common and differing selective patterns across species, along with changes in genes crucial to sperm formation and function. It has been proposed that the evolution of protamine genes takes place under the influence of sperm competition. This doctoral thesis thus focuses on an evolutionary genotype-phenotype study in mammals, examining sequence and regulatory evolution of protamines and accompanying changes in sperm head phenotype. Analyses were undertaken of protamine gene and regulatory sequence evolution as well as comparative gene expression aiming to show the importance of protamine function on sperm head size and shape. Species exhibiting different levels of sperm competition served as models to assess relationships between sperm competition and sperm head phenotype brought about by selective pressures acting on these proteins. The results presented here are important for a better understanding of the complex interplay between selective forces potentially acting on DNA sequences and cell phenotype. Gene and regulatory sequences were obtained by standard PCR techniques as well as gene walking PCR. Quantitative PCR was used to obtain gene expression data. Genotype-phenotype association studies were developed using root-to-tip dN/dS (nonsynonymous / synonymous substitutions rate ratio) to account for evolutionary rates and phylogenetic generalized least squares analyses to compare genetic and morphometric data. The results of this study revealed a surprisingly complex pattern of selective constraints and sperm competition acting on protamine sequence divergence and expression levels and, moreover, they provide compelling evidence for the effect of protamines on sperm head shape and size.

CHAPTER 2
GENERAL INTRODUCTION

Protamines are small basic sperm nuclear proteins exhibiting a very important role in reproduction. They are widely studied in humans as well as in rat and mouse models due to their impact on male fertility. Understanding the evolution, regulation and function of protamines might help advance our knowledge of evolutionary processes underlying changes in genotype and phenotype, mechanisms of gamete function in reproductive biology and unravel processes affecting male fertility. A general introduction to sperm cell biology and evolution, and the structure and role of protamines in sperm development and function, will be presented first. Then, the main aim and model species used in this study will be summarized.

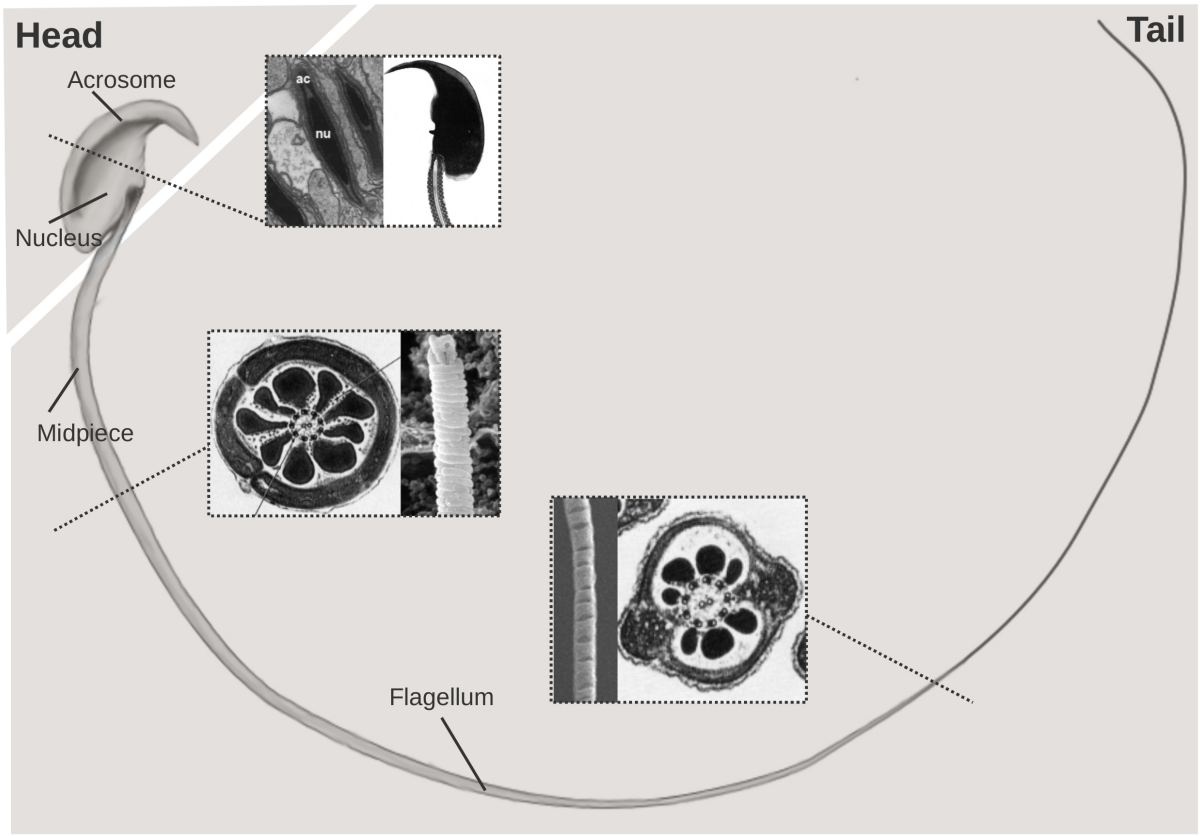


Figure 2.1. Rodent sperm cell and its components.

THE SPERM CELL. PHENOTYPE AND EVOLUTION

Spermatozoa are highly specialized, motile cells. Although sperm share a common function, that is to fertilize the female gamete, a wide variety of sperm cell morphologies has evolved in the animal kingdom (Cohen 1977). This diversity is known to be a result of selective pressures to ensure successful fertilization and ultimately the reproductive success of the male. The sperm cell consists of two main structures (Fig. 2.1):

- The sperm head containing the nucleus with the haploid paternal chromosome set and the acrosome, an exocytotic granule containing enzymes which act during the penetration and communication with the female gamete.
- The flagellum (tail), containing movement-generating structures, which consists of the midpiece, with the mitochondria that generate energy via oxidative phosphorylation (OXPHOS), and the principal piece producing energy via the glycolytic pathway.

Spermatozoa variation is present in both the flagellum and the head. Sperm without a fagellum, as well as sperm with multiple flagella, are known to exist. Sperm heads can be simple, oval-shaped or they may exhibit complex appendices. Externally fertilizing species usually show the most simple sperm architecture since the fertilizing medium is water (see Birkhead et al 2009). It is when fertilization is internal that most of the diversity can be observed. Internally fertilizing sperm need to adapt to a multitude of environments and structures and this may have led to such intense diversification (Briskie et al 1997, Pitnick et al 2003).

The sperm cells of reptiles, birds and mammalian monotremes are vermiform, while the spermatozoa of the majority of eutherian mammals show a clearly distinguished, often oval, flattened sperm head. Within Eutheria and Metatheria (marsupials), the greatest sperm diversity is found in the shape of the sperm head. The most complex cases of sperm head diversity in eutherians can be seen in rodents (Cummins and Woodall 1985, Roldan et al 1992) (Fig. 2.2). The simplest type of rodent sperm head is consistent with the ancestral eutherian form, and is described as an oval-shaped head without appendices, and with a relatively short flagellum. This sperm “design” (hereafter referred to as “bauplan”) varies greatly in the head as well as in the tail compartments among rodents. In different rodent lineages the appearance of increasingly complex sperm head appendices, such as an apical hook, and the elongation of the flagellum seems to be the general trend,

whereas some species have, in several instances, evolved a simpler sperm head similar to that seen in the eutherian sperm bauplan (Fig. 2.3). It is still not clear which selective forces drive these evolutionary patterns (Roldan et al 1992).

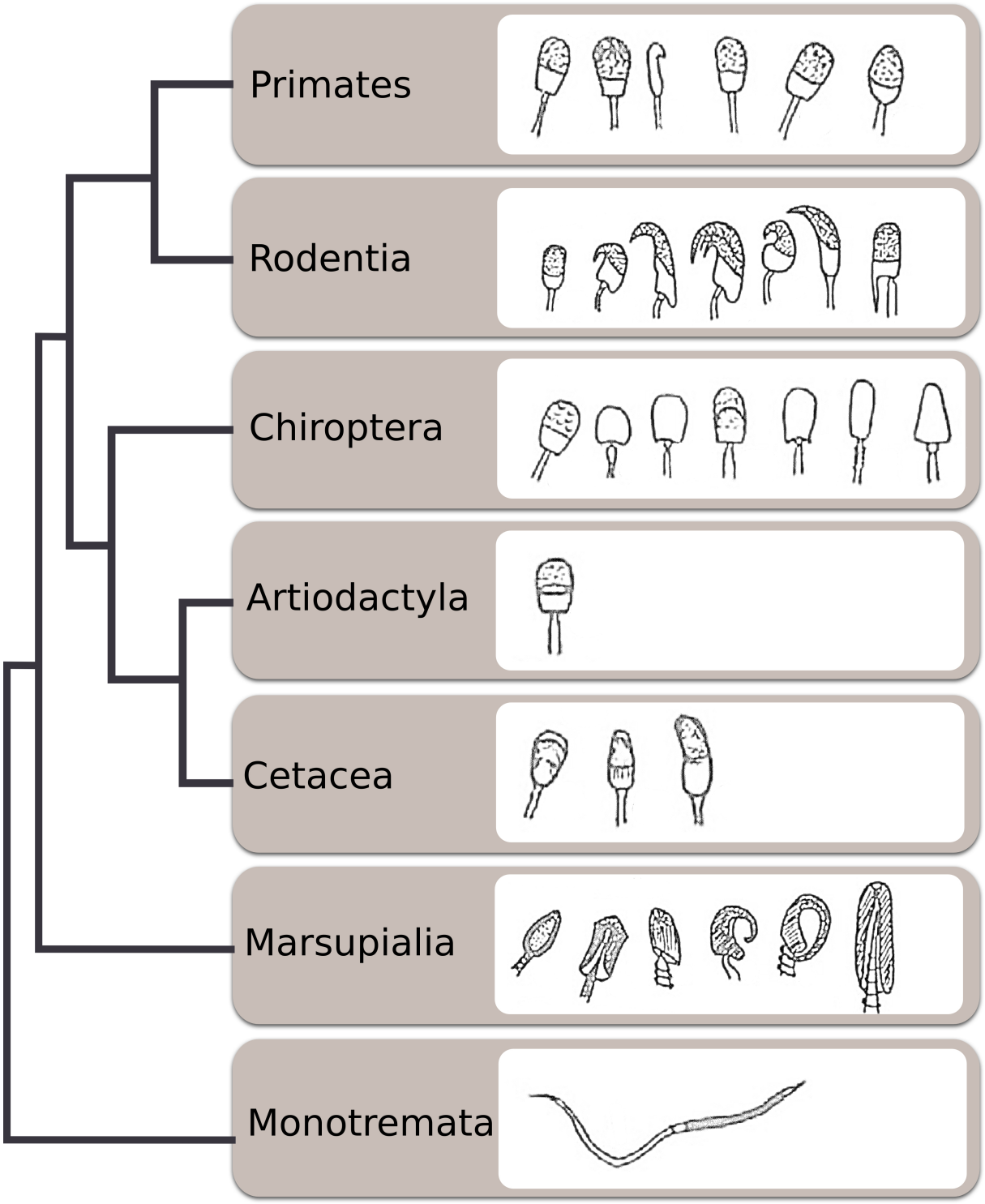


Figure 2.2. Cladogram representing morphological diversity of sperm head shape observed in mammalian clades. Modified from Roldan et al (1992).

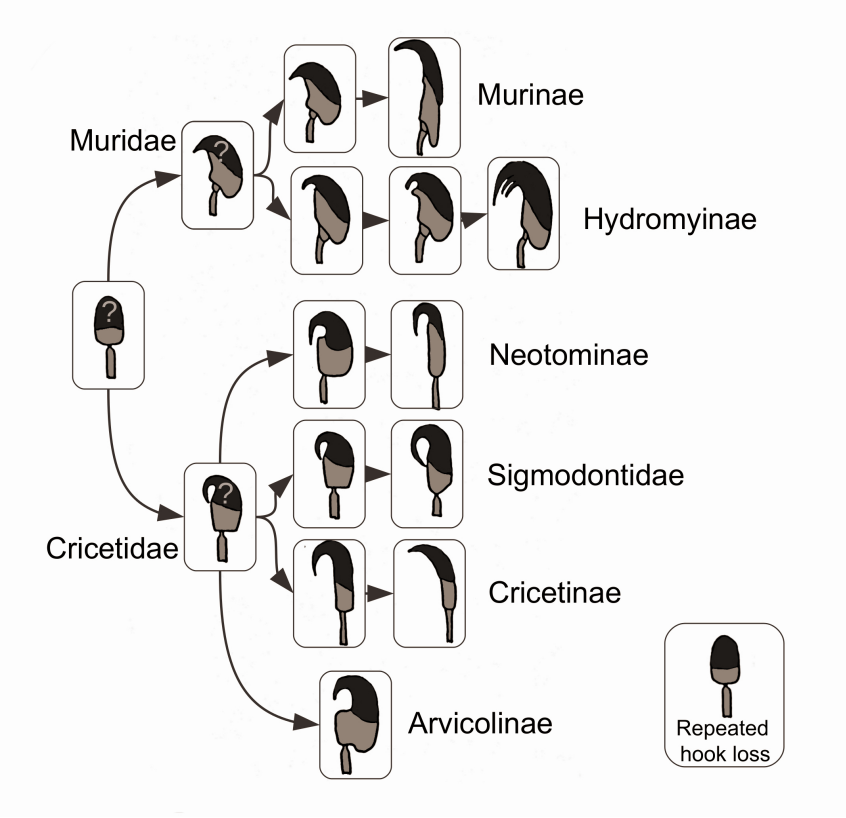


Figure 2.3. Cladogram representing evolution and diversity of rodent sperm head phenotypes. Modified from Roldan et al (1992).

SPERM DIFFERENTIATION AND UNDERLYING MOLECULAR BASIS

The process of sperm formation (spermatogenesis) can be divided into 3 phases: a mitotic proliferation phase, a meiotic phase, and a post-meiotic phase defined as spermiogenesis. Germ cells in the post-meiotic phase can be classified as early spermatids with round nuclei, intermediate spermatids with elongating nuclei, and spermatids with condensed nuclei.

During spermiogenesis important structures develop simultaneously. The acrosome is formed as a Golgi-derived organelle, and contains enzymes which play an important role in the penetration of the zona pellucida of the oocyte. The flagellum and the mid-piece develop providing the structures necessary for sperm cell movement. The cytoplasm is greatly reduced and eliminated by the supporting Sertoli cells. Nuclear DNA is compacted greatly reducing the size of the sperm nucleus (Dadoune 2003).

Sperm chromatin is radically reorganized and condensed in various steps through replacement of histones by testis-specific nuclear proteins. In the haploid round

spermatids histones are first partly replaced by a sperm-specific variant after which transition proteins are incorporated. This is followed by a stage of translational repression during which transcripts are stored. Subsequently, transition proteins are replaced by protamines during the elongating spermatid stages resulting in the compaction of sperm chromatin to a cristalline-like structure. This compaction results in nearly complete silencing of transcription and efficient protection of the paternal chromatin (Oliva and Dixon 1991, Balhorn 2007) (Fig. 2.4).

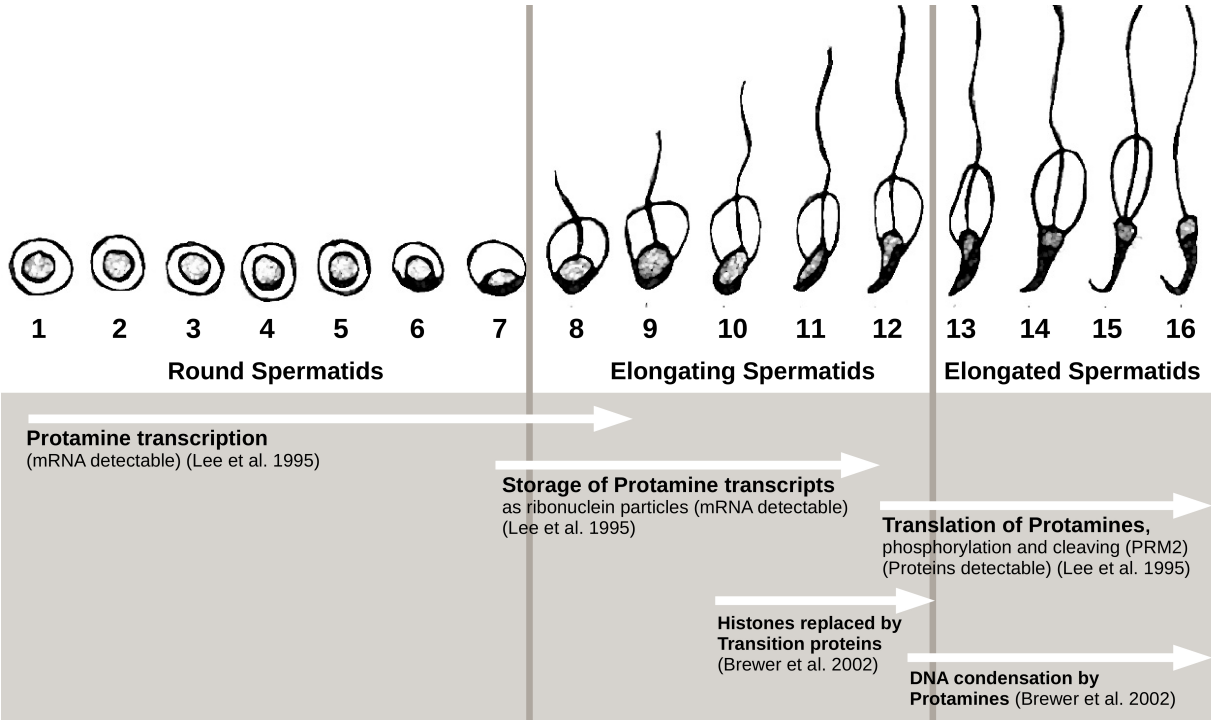


Figure 2.4. Schematic representation of rodent spermiogenesis phases and stages (numbered). Timing of protamine transcription, storage and translation as well as processing and binding are represented by arrows. Data taken from literature, References indicated in figure.

POSTCOPULATORY SEXUAL SELECTION

Sexual selection is a powerful selective force generated by the competition within and between sexes (Darwin 1871). It is now known that sexual selection does not stop with copulation but continues after mating in a process called postcopulatory sexual selection (Birkhead and Moller 1998). Postcopluatory sexual selection, in the form of sperm competition and cryptic female choice, presents a hidden, yet strong, selective force. When females mate promiscuously ejaculates of rival males need to compete in order to fertilize one ovum, or a set of ova. This process is referred to as sperm competition

(Parker 1970). The degree of female promiscuity differs greatly among species which leads to a high variation in the intensity of sperm competition and its adaptive responses to it. In this study we will focus on sperm competition, due to its proposed effect on the evolution of proteins involved in reprodutive processes and sperm phenotype.

Sperm competition is a powerful selective force known to affect many reproductive traits. An almost universal response to increased levels of sperm competition is an increase in testes size and sperm production (Gomendio et al 1998). The relationship between increased levels of sperm competition and larger relative testes mass has been widely demonstrated (Hosken and Ward 2001) and has been shown to associate to genetic paternity (Soulsbury 2010). Thus, relative testes mas has been commonly used as a proxy for levels of sperm competition. Higher relative testes mass results in elevated sperm numbers leading to an increase in sperm per ejaculate (Møller 1989). A higher sperm number at copulation increases the chances of fertilization because of the considerable sperm losses in the female tract, with only very few sperm reaching the site of fertilization in mammals as demonstrated by theoretical models and experimental studies (Parker and Pizzari 2010, Birkhead and Møller 1998).

Sperm competition also leads to adaptations of other ejaculate traits crucial for fertilization success such as sperm viability (Hunter and Birkhead 2002), normal morphology, sperm motility, and acrosome integrity. Sperm competition promotes the co-evolution of these sperm traits (Gomez Montoto et al 2011). The two sperm traits that have been most studied and are known to be affected by sperm competition are sperm bauplan (i.e., shape and size of sperm components) and sperm swimming velocity. Larger sperm size is one of these adaptations as clearly shown by several interspecific studies (Gomendio and Roldan 1991, Tourmente et al 2011) (Fig. 2.5). An elongation of the sperm head may enhance sperm swimming performance, the increase in the midpiece (which contains the mitochondria) is expected to increase energy production, and an increase in the principal piece of the flagellum could result in greater propulsive forces (Tourmente et al 2011). Associations between sperm competition levels and sperm swimming velocity have been demonstrated by several comparative studies (Gomendio and Roldan 2008, Fitzpatrick et al 2009, Gomez Montoto et al 2011). The ability of sperm to swim faster while in competition with a rival male is advantageous since the first spermatozoon that reaches the ovum is most likely to fertilize (Florman and Ducibella 2006). Sperm swimming speed is one of the main determinants of fertilization success in both non-competitive (Froman et al 1999) and competitive contexts (Birkhead et al 1999), so it is expected to be a main

target of selective pressures. How sperm can increase their swimming velocity is not well known. Changes in pathways involved in energy production might influence sperm swimming velocity as an adaptation to sperm competition (Storey 2008). Changes in sperm head shape resulting in a more hydrodynamically efficient form could be another. A strong effect of sperm head shape on sperm swimming has been demonstrated in rodents (Gómez Montoto et al 2011) (Fig. 2.6). How sperm competition affects sperm head development and bauplan through selective pressures on gene and regulatory sequences and gene expression, specifically on protamines, is the main focus of study presented here.

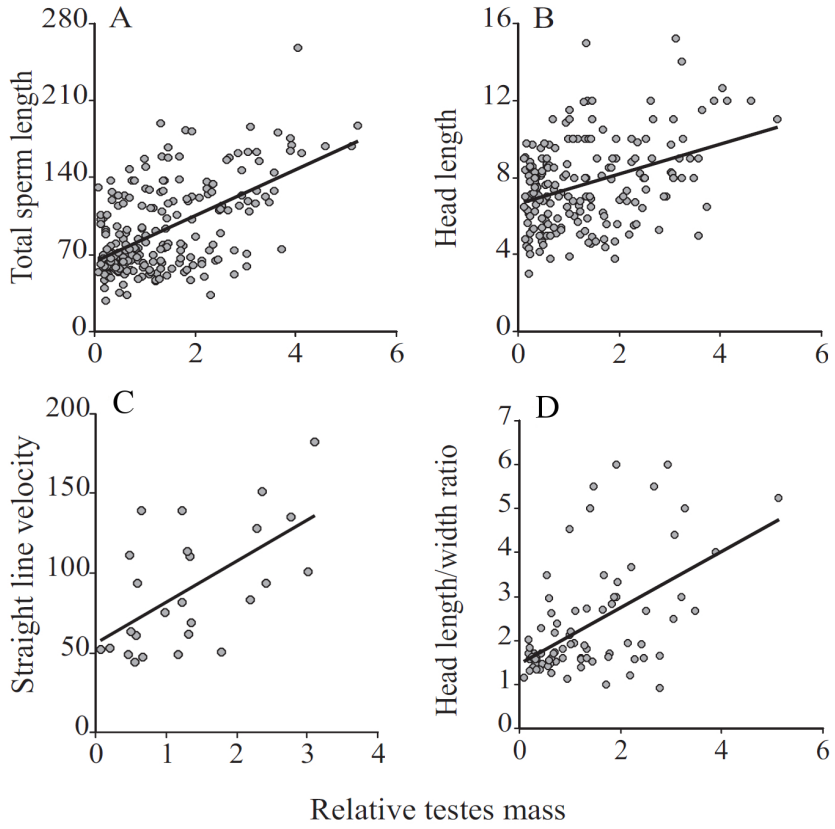


Figure 2.5. Effect of sperm competition on sperm dimensions and sperm velocity in eutherian mammals. Relations between relative testes mass and (A) total sperm length (μm), (B) sperm head length (μm), (C) Sperm straight-line swimming velocity (μm/s), and (D) sperm head length/width ratio.
Taken from Tourmente et al (2011)

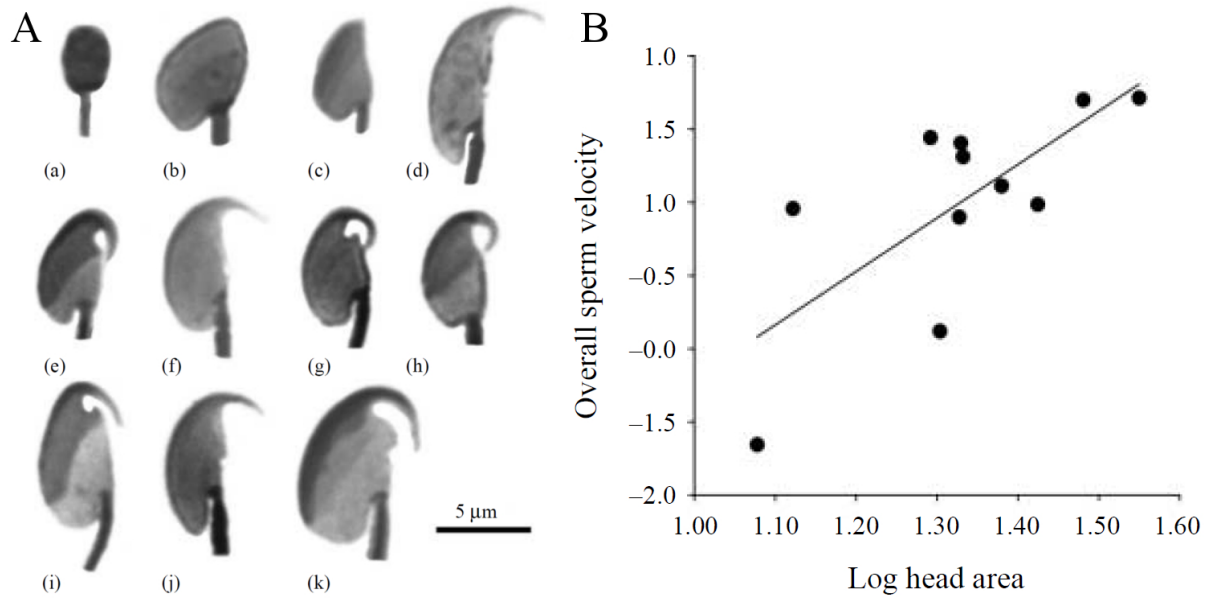


Figure 2.6. Effect of sperm head morphology on sperm swimming velocity. (A) Sperm head morphology in muroid species. (a) *Microtus duodecimcostatus*, (b) *Microtus cabrerae*, (c) *Microtus lusitanicus*, (d) *Mus musculus*, (e) *Microtus arvalis*, (f) *Mus spretus*, (g) *Arvicola sapidus*, (h) *Clethrionomys glareolus*, (i) *Chionomys nivalis*, (j) *Mus spicilegus* and (k) *Apodemus sylvaticus*. (B) Relationship between sperm head area and overall sperm velocity in muroid rodents. Modified from Gómez Montoto et al (2011)

REPRODUCTIVE PROTEINS AND SPERM COMPETITION

Proteins of the reproductive system that affect male and female traits are thought to experience rapid divergence in their gene sequences (Swanson and Vacquier 2002, Turner and Hoekstra 2008). However, it has been shown that this connection is not as straightforward as assumed. Evolutionary rates of reproductive proteins seem to vary according to their involvement in reproductive processes or localization of expression (Dorus and Karr 2009, Findlay and Swanson 2010, Vicens et al 2014).

The effect of postcopulatory sexual selection on evolutionary rate of these proteins has been widely studied. Yet, relatively few studies have been able demonstrate this relationship. The evolutionary rate of the coding sequences of two seminal fluid proteins SEMG2 and SVS and of proteins expressed on the sperm surface (ADAM 2 and ADAM18) and the acrosome (Zonadhesin, SPAM1) have been found to be positively affected by postcopulatory sexual selection in primates (Dorus et al 2004, Ramm et al 2008, Finn and Civetta 2010, Herlyn and Zischler 2007, Prothmann et al 2012). Other studies found a negative effect of sperm competition on the gene sequence divergence of seminal fluid

proteins in butterflies or protamine 1 and protamine 2 in rodents (Walters and Harrison 2011, Lüke et al 2011).

PROTAMINES

Protamines are a diverse family of small, arginine-rich nuclear proteins that replace histones and transition proteins during the process of sperm nucleus condensation in the final stages of spermatogenesis (Oliva and Dixon 1991). The high charge density of these proteins allows them to bind DNA with high affinity and to more efficiently shield the charges on the DNA phosphate backbone than histones. This charge neutralization results in a genetically inactivate state of most of the spermatid genome and in the reduction of the size of the sperm nucleus to a minimum (Balhorn 1989, Tanaka and Baba 2005). Expression of protamines is specific to testis and protamine mRNAs are exclusively detected in postmeiotic spermatid stages (Lee et al 1995). Protamines first appear in elongating spermatids, coinciding with the initiation of the final stage of chromatin condensation (Lee et al 1995, Brewer et al 2002) (Fig. 2.4).

Two types of protamines have been identified: protamine 1 (*Prm*1, PRM1) and protamine 2 (*Prm*2, PRM2). While PRM1 is a major sperm protamine present in all mammals, PRM2 is present only in the sperm of primates, most rodents, and a subset of other placental mammals (Chauviere et al 1992, Retief and Dixson 1993) (Fig. 2.7). It has been proposed that *Prm2* is the result of a *Prm*1 duplication event (Krawetz and Dixon 1988, Lüke et al 2011). PRM1 is usually 49 or 50 amino acids long and contains 3 or more DNA anchoring domains consisting of 3-7 arginine residues separated by uncharged amino acids as well as multiple serine and threonine residues as possible phosphorylation sites (Balhorn et al 1999, Balhorn 2007). PRM2 exhibits similar structural and functional properties as PRM1. However, *Prm*2 codes for a PRM2 precursor, termed pre-PRM2. PRM1, in contrast, is not synthesized as a precursor (Retief et al 1993). The pre-PRM2 is processed in late-spermatids by proteolytic cleavages in its N-terminal region (hereafter "cleaved PRM2") (Yelick et al 1987, Oliva and Dixon 1991, Retief and Dixon 1993). The fully processed form of the PRM2 (hereafter "mature PRM2") typically consists of 63 amino acids in the mouse and is the predominant form of PRM2 in the head of mature sperm. Cysteine residues in protamines of eutherian mammals allow for the formation of disulphide bonds within and between protamines stabilizing the chromatin structure (Balhorn et al 1992, Queralt et al

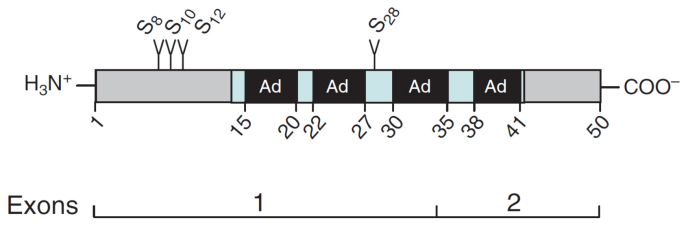
1995). In marsupials, PRM1 cysteine residues can only be found in the *Planingale* genus.

It has been shown in several studies on human and mouse models that alterations in protamine expression can affect fertility (Oliva 2006, Cho et al 2003, Aoki et al 2005, Carrell et al 2007). Alterations in sperm protamine content negatively affects sperm number, motility and sperm head morphology in men (Aoki et al 2005) and causes morphological abnormalities, DNA damage, and reduced sperm motility in mice (Cho et al 2001). Interestingly the protamine ratio (PRM1/PRM2) has been described to vary greatly between mammalian species (0-70% PRM2) but is nearly constant within the same genus (Corzett et al 2002). Although the reason is still not very well understood, alterations of the species-specific protamines ratios are important for proper sperm differentiation and seem to be associated to male infertility even more so than absolute protamine amounts (Balhorn et al 1988, Steger et al 2001, 2003) (Fig. 2.8).

In agreement with their known role in DNA compaction, altered protamine content leads to inadequate sperm chromatin condensation resulting in enlarged sperm heads as well as head abnormalities (Belokopytova et al 1993). The binding of protamines to DNA in elongating spermatids results in the coiling of DNA in a toroidal structure (Ward and Coffey 1991). The resulting structure is highly compact. The exact mechanism of protamine-mediated DNA compaction is still being studied but it has been proposed that due to the attachment of DNA to an inner nuclear matrix (MAR) the strong compaction of the DNA through protamines can generate enough force to reshape the sperm head (Cree et al 2011) (Fig. 2.9). This effect on sperm head shape may influence hydrodynamic efficiency of the sperm resulting in an increase in sperm swimming speed and more competitive sperm.

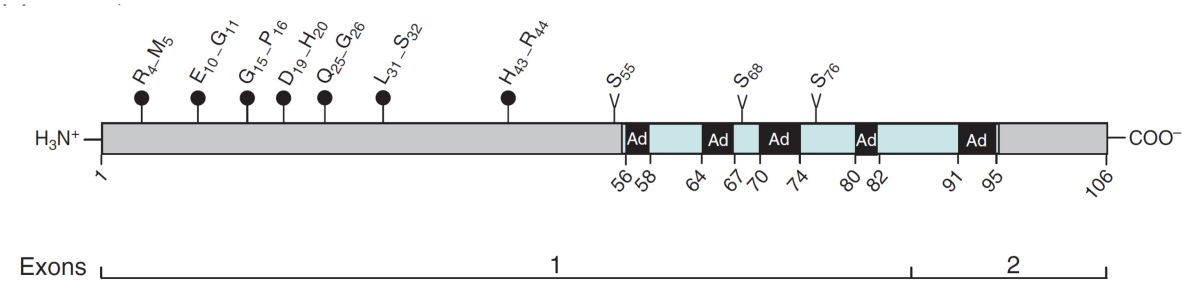
A Protamine 1 (*Mus musculus*)

MARYRCCRSKSRSRCRRRRRRCRRRRRRCCRRRRRRCCRRRRSYTIRCKKY



B Protamine 2 (*Mus musculus*)

MVRYRMRSPSEGPHQGPGQDHEREEQGQGQGLSPERVEDYGRTHRGHHHHR
HRRCSRKRLHRIHKRRRSCRRRRRHSCRHRRRHRRGCRRSRRRRRCRCRKCR
RHHH



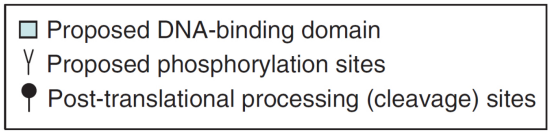


Figure 2.7. Protamine amino acid sequences, domains and modification sites. Cleaved-PRM2 amino acid sequence is shown in grey font, mature-PRM2 and PRM1 amino acid sequences are shown in black font. Adapted from Balhorn (2007).

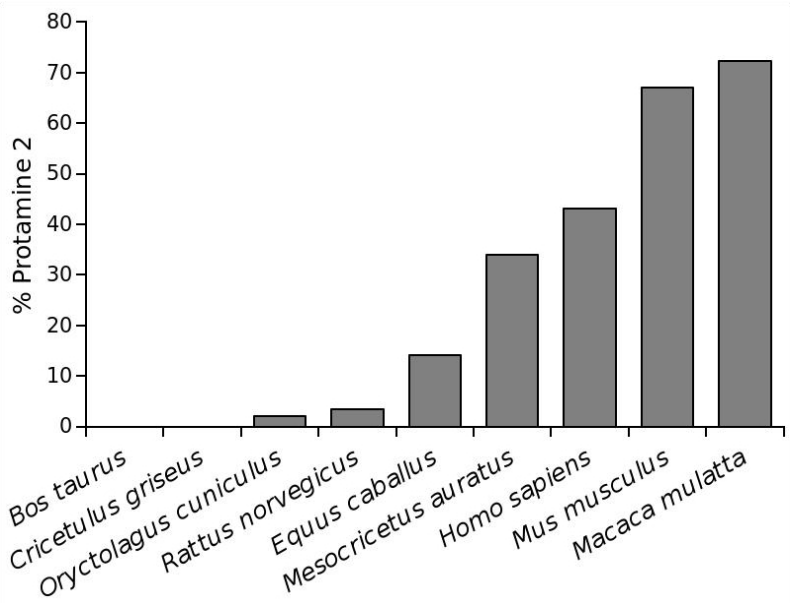


Figure 2.8. Comparison of relative protamine expression in mammals. Represented as percentage of protamine 2 of total protamine expression. Data for graphical representation taken from Corzett et al (2002).

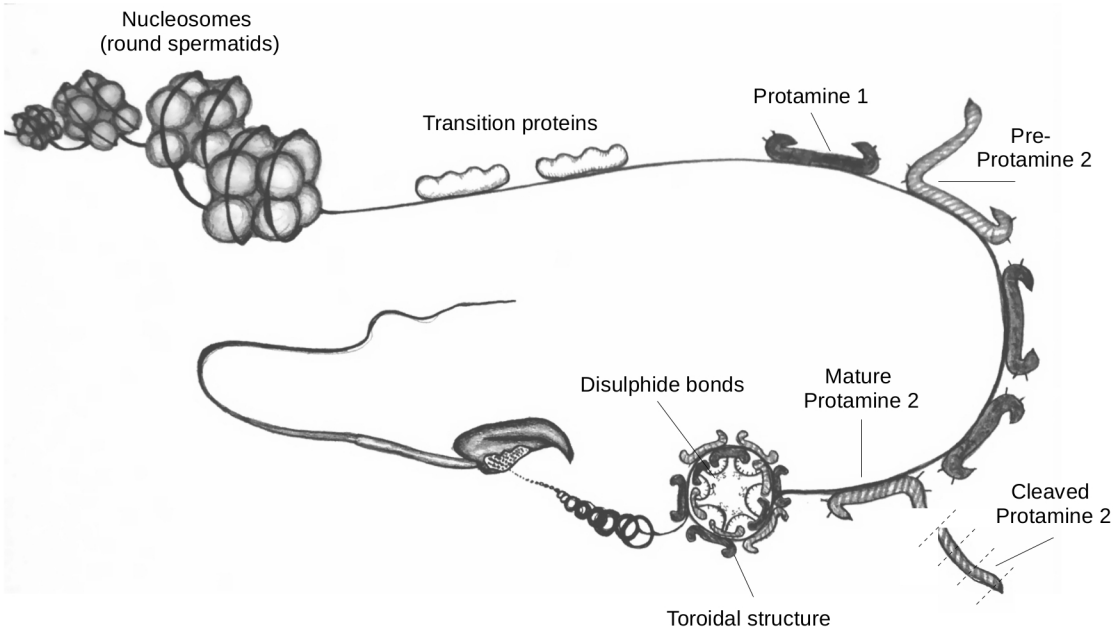


Figure 2.9. Schematic representation of protamine-mediated DNA condensation.

EVOLUTION OF PROTAMINES

Protamines are thought to evolve rapidly, displaying high structural heterogenity in comparison to other sperm nuclear basic proteins. Sexual selection is thought to be the underlying force (Oliva and Dixon 1991, Wyckoff et al 2000). Still, evidence of purifying selection or sequence conservation can be found as well. Selective conservation of the high arginine concentration exists while the position of arginine residues seems to be variable. Even though within mammals protamines have been shown to contain conserved regions which are also found in birds (N-terminal ARYR, SRSRSR phosphorylation site, 3 arginine clusters) they are generally diverse, especially in the C-terminal region (Queralt et al 1993). Evidence of positive selection on protamines has been detected in primates (Rooney and Zhang 1999, Wyckoff et al 2000) while other studies have demonstrated different selective constraints in other mammalian species (Martin-Coello et al 2009, Lüke et al 2011) (Fig. 2.10).

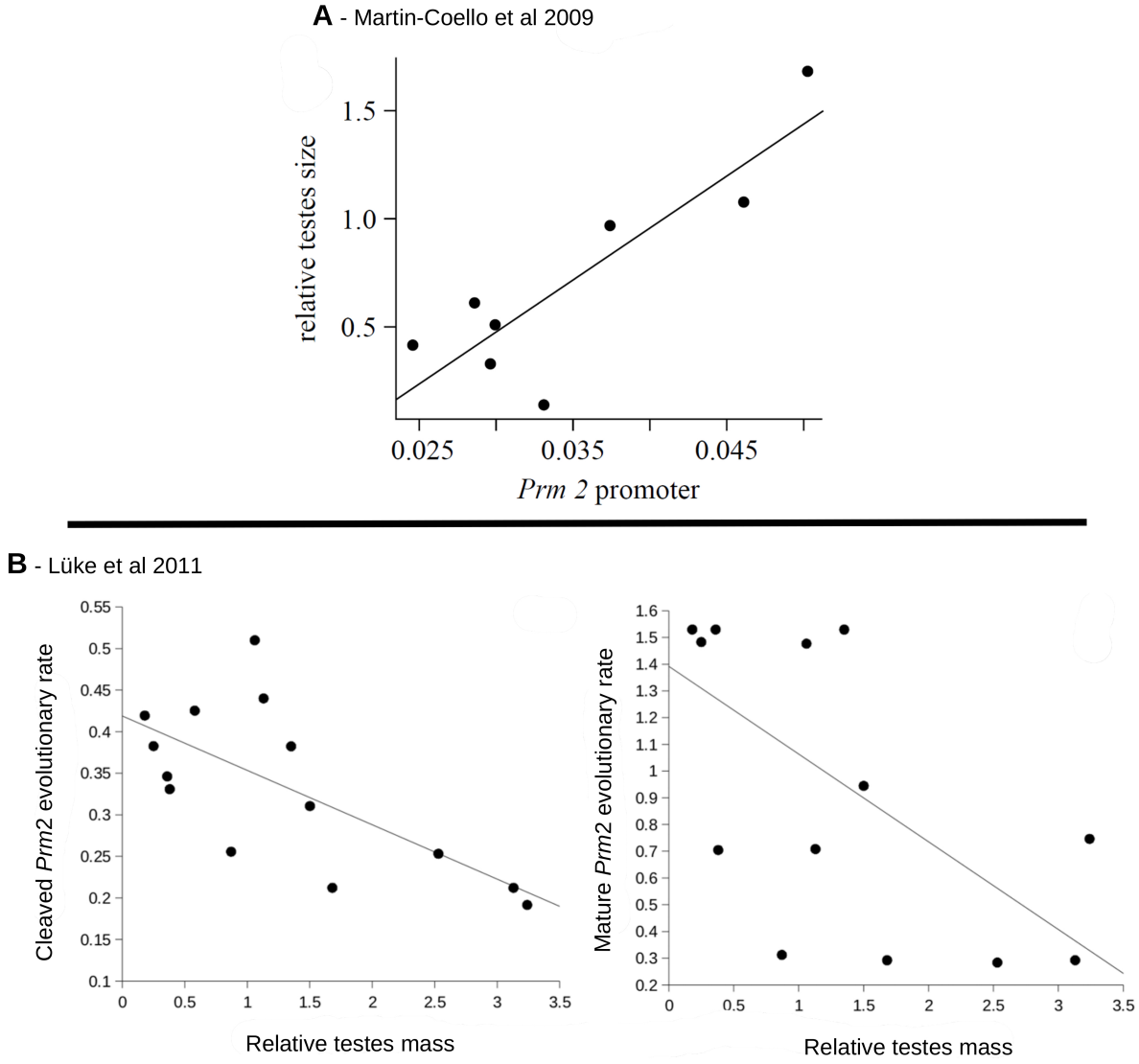


Figure 2.10. Results of previously published analyses of protamine selection. (A) Effect of sperm competition (relative testes size) on *Prm*2 promoter sequence divergence in mouse species (Martin-Coello et al 2009, (B) Effect of sperm competition (relative testes mass) on mature and cleaved *Prm*2 sequence divergence in cricetid rodents (Lüke et al 2011).

GENOTYPE - PHENOTYPE ASSOCIATION STUDIES

How the phenotype evolves through changes in genotype is a subject commanding considerable interest although it is still poorly understood. Knowledge on the connections between genotype and phenotype is crucial to uncover the processes of development and evolution but also the genetic basis of disease. In order to study the function of a gene, or sequence, and its effect on a specific phenotype, the most common approach is reverse genetics. Once candidate genes are detected, and chosen gene products are disrupted or modified (e.g., over-expressed, knocked-out), an evaluation of the phenotypic outcomes follows. Even though this experimental strategy is the most direct one, it often overstates the phenotypic outcome resulting in non functionality and false positives (Gilchrist and Haughn 2005). In these cases a different approach might be more advantageous (Salanti et al 2005).

If an appropriate, naturally-occuring evolutionary model is available, genotype-phenotype connections can be described by observation and statistical evaluation of the existing diversity and selective pressures. This approach allows for a more fine-tuned evaluation of the phenotype affected by genotype diversity and the selective pressure potentially underlying them. A subsequent confirmation and comparison of the associations uncovered by means of a modification of the gene product, and subsequent observation of the phenotypic outcome, would then lead to a very well-supported and properly-interpreted result.

ANIMAL MODELS

In order to study and compare evolutionary patterns of protamine gene sequences we chose species from a broad range of mammalian clades for which gene sequence information is avaible in public databases. Since *Prm*1 is present in all mammals we were able to include both marsupial and placental mammals in the evolutionary study of this gene. In the study of the *Prm*2 gene sequence we focused on a direct comparison of rodents and primates because *Prm*2 is mainly found in these mammalian lineages and little is known about the presence and role of *Prm2* in other mammals (Chauviere et al 1992, Retief and Dixson 1993).

In order to analyze the evolution of regulatory sequences and gene expression, with

accompanying changes of sperm head phenotype, we concentrated on rodent species for several reasons. First, sperm head phenotype in rodents has been shown to be particularly diverse (Cummins and Woodall 1985, Roldan et al 1992). Second, rodents express both PRM1 and PRM2 (Chauviere et al 1992, Retief and Dixson 1993). Third, rodent species exhibit a wide range of sperm competition levels. Fourth, rodent reproductive phenotype shows clear responses to sperm competition and, with regards to their variation in sperm size, they do not seem to be constrained by metabolic rate. Fifth, a genetic characterization is well established for these species.

Within the rodent clade we focused on three subfamilies for detailed study: Murinae (mice), Cricetinae (hamsters) and Arvicolinae (voles). These three taxa stand out due to their marked differences in sperm head shape despite their close taxonomic relation (Varea Sanchez 2014). The subfamily of mice emerged 5 millions years ago (MYA), covers a broad range of sperm competition levels but relatively low diversity in protamine gene sequences. The family of voles and hamsters (Cricetidae) emerged 18 MYA and therefore experienced a longer diversification time and show higher diversity in protamine gene sequences (Steppan et al 2004, Martin-Coello et al 2009, Lüke et al 2011). Voles cover a range of sperm competition levels similar to mice, while hamsters generally show higher levels than both voles and mice (Martin-Coello et al 2009, Lüke et al 2011) (Fig. 2.11).

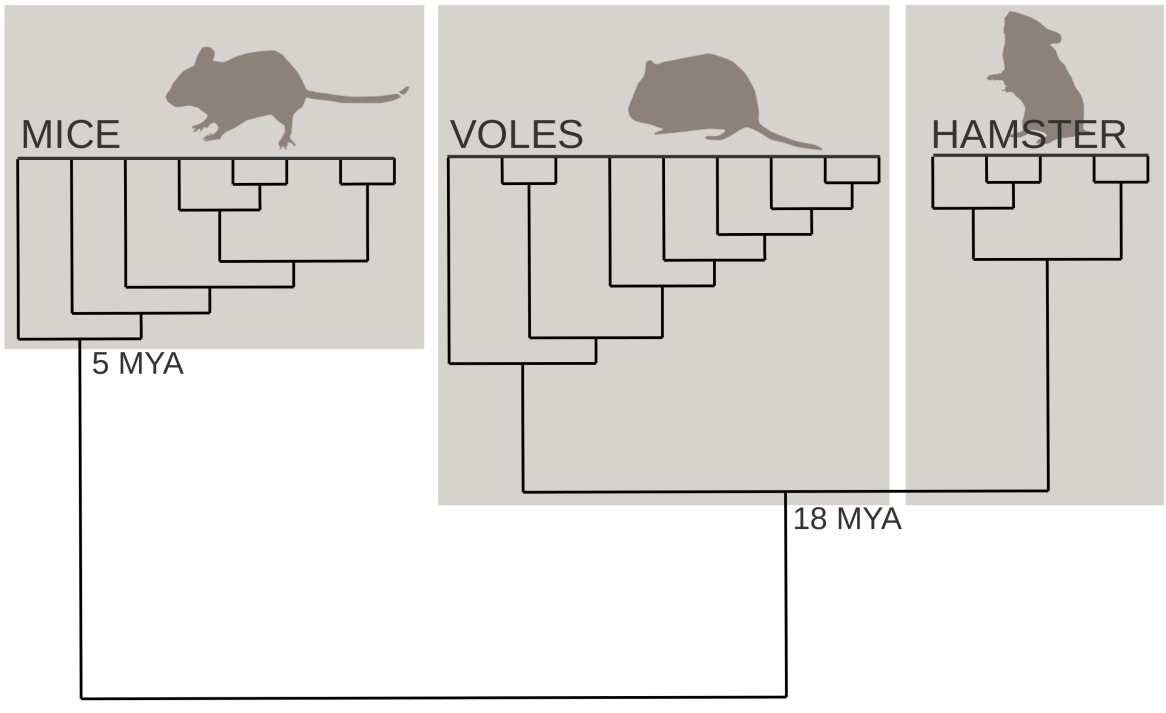


Figure 2.11. Subfamilies of rodent families analysed. MYA = million years ago. Divergence time according to Steppan et al (2004).

HYPOTHETICAL ASSOCIATIONS BETWEEN SPERM COMPETITION, PROTAMINES AND SPERM HEAD PHENOTYPE

In this study we examine protamines in detail using an evolutionary approach not only to unravel their function and impact on phenotype but also as a "proof-of-concept" aiming to set up an approach to understand the evolutionary processes affecting reproductive traits and function at the molecular and phenotypic levels. We analyze how postcopulatory sexual selection, in the form of sperm competition, might lead to an adaptation of sperm head phenotype through modification of protamines. To this end we elaborated a set of hypotheses and examined a series of predictions related to possible associations (Fig. 2.12).

Due to their role in male fertility, sperm cell differentiation and sperm head shaping, we predicted that sperm competition affects protamine sequence and regulatory evolution as well as expression levels. Species exhibiting high levels of sperm competition may exhibit adaptations of sperm head phenotype as a result of selective pressures acting on protamine coding sequence, amino acid composition, regulatory sequences and expression levels. We anticipated associations between sperm competition level and protamines as well as between sperm competition level and sperm head phenotype. Additionally we expected to find evidence for the effect of protamines on sperm head phenotype resulting in a extended link in the relationship between sperm competition – protamines – sperm head phenotype.

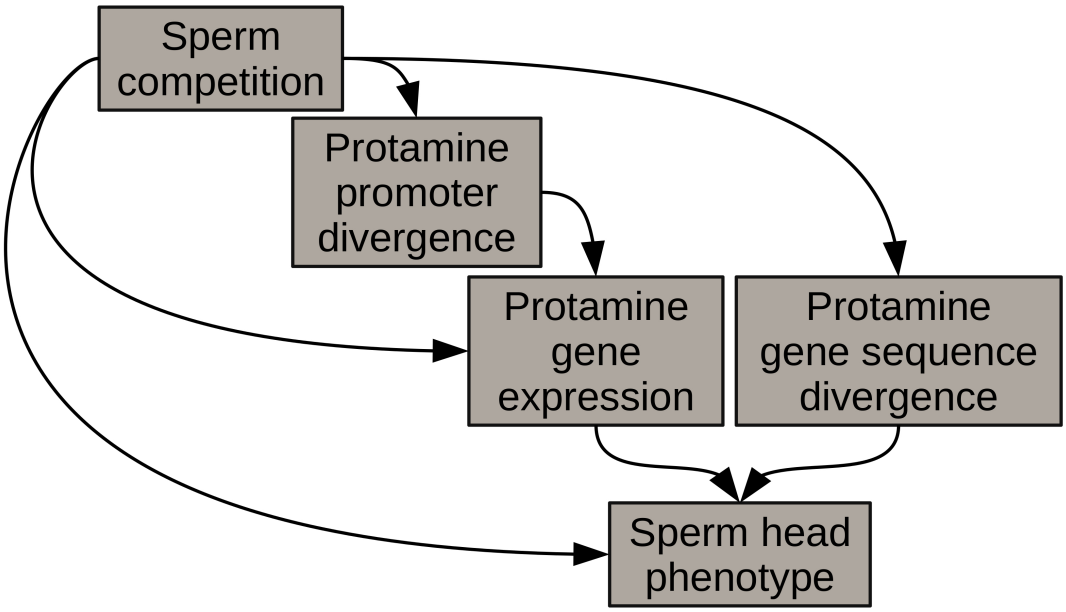


Figure 2.12. Hypothetical associations between sperm competition, protamines and sperm head phenotype.

CHAPTER 3
OBJETIVOS

El objetivo general de esta tesis doctoral fue el estudio de la evolución y la función de las protaminas mediante un estudio comparativo. El propósito fue analizar los patrones evolutivos y de selección en mamíferos y, en forma subsiguiente, focalizar los análisis en especies modelo que exhiben una diversidad en la morfología de los espermatozoides y diferentes niveles de competición espermática. Este estudio se diseñó con un enfoque integrador con el fin de identificar estrategias para futuros análisis de relaciones genotipo-fenotipo, y para la caracterización de procesos selectivos que modelan el fenotipo a través de cambios genotípicos.

Los objetivos específicos del estudio fueron:

1. Determinar si, y cómo, la selección sexual, del tipo de competición espermática, afecta a la evolución molecular de las protaminas en mamíferos.
2. Comparar los patrones evolutivos y de selección de las protaminas 1 y 2 en primates y en roedores.
3. Comparar los patrones evolutivos y de selección de las protaminas en mamíferos con el fin de identificar posibles tendencias evolutivas comunes.
4. Obtener conclusiones sobre función de protaminas mediante análisis detallado de patrones evolutivos y de selección que actúan sobre las secuencias codificantes de las proteinas y su expresión.
5. Examinar las relaciones entre protaminas y el fenotipo de la cabeza espermática mediante un análisis detallado de patrones de evolución y selección que actúan sobre el genotipo en especies con gran diversidad de morfologías espermáticas.
6. Analizar cómo la selección sexual puede modelar el fenotipo reproductivo a través de cambios en genotipo, empleando un estudio evolutivo de asociación genotipo-fenotipo de las protaminas en un modelo de roedores.

OBJECTIVES

The main objective of this doctoral dissertation was the study of protamine evolution and function through a comparative analysis. We aimed to analyze evolutionary and selective patterns throughout mammals and subsequently focus our analysis on model species exhibiting morphological sperm diversity and varying sperm competition levels. We designed this study with an integrative approach in order to identify means for future genotype-phenotype analyses of other reproductive proteins, and the characterization of selective processes shaping phenotype through genotype.

The specific objectives were to:

1. Determine if and how sexual selection, in the form of sperm competition, affects the molecular evolution of protamines in mammals.
2. Compare evolutionary and selective patterns of protamines 1 and 2 in primates and rodents.
3. Compare evolutionary and selective patterns of protamines among mammals determining a possible common evolutionary trend.
4. Draw conclusions about protamine function through detailed analysis of evolutionary pattern and selection acting on protamine coding sequences and expression.
5. Assess the relationship between protamines and sperm head phenotype through a detailed analysis of evolutionary and selection patterns acting on protamine genotype in species exhibiting highly diverse sperm head morphologies.
6. Analyze how sexual selection may shape reproductive phenotype through changes in genotype, employing an evolutionary genotype-phenotype association study of protamines in a rodent model.

CHAPTER 4

EVOLUTION OF PROTAMINE GENES AND CHANGES IN SPERM HEAD PHENOTYPE IN RODENTS

RESUMEN

Poco se sabe sobre las bases genéticas de los cambios evolutivos en el fenotipo espermático. La selección sexual postcópula se asocia con diferencias en las secuencias génicas y de los promotores de las protaminas, y es una fuerza selectiva potente que actúa sobre la forma y función de los espermatozoides, aunque existe evidencia limitada de las relaciones entre la evolución de las protaminas y el fenotipo espermático. Las protaminas están involucradas en la condensación de la cromatina espermática. Las deficiencias en las protaminas afectan negativamente la forma de los espermatozoides y la fertilidad masculina, sugiriendo de este modo que son importantes para el diseño y la función espermática. En el presente estudio se examinaron cambios en los genes de las protaminas y el fenotipo espermático en roedores para comprender el papel de la selección sexual en la evolución de las protaminas y el diseño de las celulas espermáticas. Se realizó un estudio de asociación genotipo-fenotipo, empleando un "ratio dN/dS desde la base al extemo" para calcular las tasas evolutivas, y un analisis filogenético generalizado de cuadrados mínimos para comparar datos genéticos y morfométricos. Las tasas evolutivas de protamina 1 y del dominio de protamina 2 que se cliva y elimina durante la condensación de la cromatina correlacionaron con el tamaño y la elongación de la cabeza espermática. La protamina 1 exhibió selección positiva restringida a algunos sitios funcionales, que parecía ser suficiente para preservar su papel en el diseño de la cabeza espermática. La protamina 2 clivada, cuya relajación es detenida por la selección sexual, parece asegurar cabezas espermáticas pequeñas y elongadas, que harían que los espermatozoides fuesen más competitivos. No se encontró asociación entre la protamina 2 madura y el fenotipo de la cabeza, sugiriendo poca participación en la condensación y un papel más probable en el estado ya condensado de la cromatina. Nuestros resultados sugieren que los cambios evolutivos en las protaminas

podrían estar relacionados con modificaciones complejas en el desarrollo de la cabeza espermática. Estos hallazgos representan un importante paso hacia una mejor comprensión del papel de los cambios en las secuencias codificantes de los genes en la divergencia del fenotipo de la célula germinal.

SUMMARY

Little is known about the genetic basis of evolutionary changes in sperm phenotype. Postcopulatory sexual selection associates with differences in protamine gene sequences and promoters, and is a powerful force acting on sperm form and function, although links between protamine evolution and sperm phenotype are scarce. Protamines are involved in sperm chromatin condensation, and protamine deficiency negatively affects sperm morphology and male fertility, thus suggesting that they are important for sperm design and function. We examined changes in protamine genes and sperm phenotype in rodents to understand the role of sexual selection on protamine evolution and sperm design. We performed a genotype-phenotype association study, using root-to-tip dN/dS to account for evolutionary rates, and phylogenetic generalized least squares analyses to compare genetic and morphometric data. Evolutionary rates of protamine 1 and the protamine 2 domain cleaved-off during chromatin condensation correlated with head size and elongation. Protamine 1 exhibited restricted positive selection on some functional sites, which seemed sufficient to preserve its role in head design. The cleaved-protamine 2, whose relaxation is halted by sexual selection, seems to ensure small, elongated heads that would make sperm more competitive. No association existed between mature-protamine 2 and head phenotype, suggesting little involvement during chromatin condensation and a likely role maintaining the condensed state. Our results suggest that evolutionary changes in protamines could be related to complex developmental modifications in the sperm head. This represents an important step towards understanding the role of changes in gene coding sequences in the divergence of germ cell phenotype.

INTRODUCTION

Postcopulatory sexual selection, in the form of sperm competition, is an evolutionary force known to drive rapid adaptation of reproductive traits (Parker 170, Birkhead and Møller 1998, Simmons 2001, Birkhead et al 2009), and that has also been linked to rapid diversification of coding sequences of the so-called reproductive proteins (Swanson and Vacquier 2002, Turner and Hoekstra 2008). Sperm competition occurs when females mate with more than one male during their receptive period, resulting in rival ejaculates competing for fertilization of ova (Parker 1970). The diversity of male reproductive traits, and the adaptive significance of differences in sperm form and function has been analyzed using comparative methods and, more recently, experimental evolution studies (Birkhead et al 2009). However, the genetic basis of evolutionary changes in sperm phenotype has, so far, received very limited attention in mammals.

The most widely recognized phenotypic response to an increased level of sperm competition is an increase in sperm numbers (Birkhead and Møller 1998, Birkhead et al 2009), which enhances a male's chance of fertilization (Birkhead and Møller 1998,Suarez et al 1990, Parker and Pizarri 2010). High sperm numbers may be achieved by an increase in the size of the testes relative to body mass and such increase associates with sperm competition in a variety of taxa (Birkhead and Møller 1998, Gomendio et al 1998, Birkhead et al 2009), including mammals (Soulsbury 2010). Another important sperm trait found to be driven by sperm competition in mammals is sperm design (i.e., sperm dimensions and head shape)(Gomendio and Roldan 1991, Immler et al 2007, Tourmente et al 2011a,b). Sperm design is known to influence sperm function by affecting sperm swimming velocity in many taxa (reviewed in Gomendio and Roldan 2008). In mammals, sperm head shape and size may infuence hydrodynamic efficiency of the sperm cell and thereby affect swimming velocity (Roldan et al 1992, Tourmente et al 2011b). Sperm that have more elongated heads and heads that are smaller in relation to the length of the flagellum exhibit higher swimming velocity. A smaller and more elongated head is believed to produce less drag during swimming and is therefore thought to positively influence sperm velocity (Humphries et al 2008). In rodents, spermatoza from many species display a hook in their rostral region; hook shape and size may also have an impact on sperm swimming velocity (Gómez Montoto et al 2011a). Altogether, the evidence available indicates that sperm head shape and size are important factors in sperm competition as they strongly influence how fast sperm may swim towards ova.

Proteins of the reproductive system that affect crucial phenotypic traits are thought to experience rapid divergence in their coding sequences (Swanson and Vacquier 2002, Turner and Hoekstra 2008). Evidence for this hypothesis is, however, limited. Only three studies have found positive relationships between gene sequence divergence and levels of sperm competition (SEMG2: Dorus et al 2004, SVS2: Ramm et al 2008, ADAM2 and ADAM18: Finn and Civetta 2010). Other studies suggest that relaxed selective constraint may also be responsible for fast evolutionary rates (Walters and Harrison 2011, Lüke et al 2011).

Protamines are nuclear proteins involved in sperm chromatin condensation. They replace histones and transition proteins during the process of sperm DNA condensation that takes place in spermiogenesis (Oliva and Dixon 1991). In some eutherian mammals two types of protamines are found: protamine 1 (PRM1) and protamine 2 (PRM2). While PRM1 is present in all mammals, PRM2 is only detected in sperm of primates, most rodents, and a subset of other placental mammals (Oliva 2006, Balhorn 2007). In contrast to the *Prm1* gene, the *Prm2* gene codes for a precursor (hereafter, "PRM2 precursor"), which is processed in elongated spermatids by successive proteolytic cleavages. This results in the removal of about 40% of the protein N-terminal region (the removed domain is hereafter referred to as "cleaved-PRM2") (Oliva and Dixon 1991, Oliva 2006). The PRM2 form resulting after full cleavage (herafter, "mature-PRM2") consists of 63 amino acids in the mouse. The mature-PRM2 shows very similar structural and functional properties to PRM1 and is proposed to be the result of gene duplication, while cleaved-PRM2 shows no resemblance and might be of retroviral origin (Krawetz and Dixon 1988 , Lüke et al 2011).

It has been claimed that protamines are the fastest evolving reproductive proteins, with sexual selection being the underlying selective force (Wyckoff et al 2000). Evidence of positive selection in PRM1 has been detected in primates (Rooney and Zhang 1999, Wyckoff et al 2000) but recent studies have revealed some contrasting results in other mammalian species. In closely related mouse species (genus *Mus*), no evidence of positive selection was found for PRM1 and only weak positive selection for PRM2 (Martin-Coello et al 2009). In a group of more diverse rodents (voles and hamsters) PRM1 is conserved with signs of localized positive selection but no evidence of being sexually selected (Lüke et al 2011). On the other hand, the *Prm2* gene sequence of voles and hamsters was found to be under relaxation, leading to degradation of the gene. This process is halted by sexual selection removing deleterious mutations in species with higher levels of sperm competition (Lüke et al 2011). In closely related species, among

which conservation of coding regions may exist, the evolution of gene regulatory regions may be an early stage in speciation. Among protamines, an association was found between the divergence of *Prm2* promoters and levels of sperm competition of mouse species (Martin-Coello et al 2009). Divergence in the *Prm2* promoter was also associated to sperm swimming velocity (Martin-Coello et al 2009), which suggests that changes in regulatory regions could increase the efficiency of DNA condensation in the sperm head, thus affecting head shape and size and, in turn, sperm performance.

The importance of protamines in sperm chromatin condensation and the influence of protein expression on sperm function has been shown in mouse and human models (Oliva 2006, Cho et al 2003, Aoki et al 200, Carrell et al 2007). Alterations in sperm protamine content can have major negative effects on sperm concentration, motilty and sperm head morphology in men (Aoki et al 2005). Haploinsufficiency of protamines in mice results in sperm morphological abnormalities, DNA damage and decreases in sperm motility (Cho et al 2001). In particular, PRM2 deficiency has a negative impact on chromatin packaging and sperm head morphology (Cho et al 2003). Incorrect condensation of sperm chromatin results in larger heads as well as head abnormalities (Belokopytova et al 1993). This further supports the idea that protamines are important in sperm head formation and function.

To understand the effect of sperm competition on reproductive trait evolution both molecular changes and the phenotypic response should ideally be analyzed together. Here we analyze the effect of protamines on sperm head size by examining a possible association between divergence in gene sequences and various sperm head dimensions. In order to compare genetic and morphometric data we employed methods used in previous studies to analyze genotype-phenotype correlations, i.e., the phylogenetic generalized least squares approach using phylogenetic data and root-to-tip dN/dS (Montgomery et al 2011, Pointer et al 2012). Using these approaches, genetic-morphometric associations have been uncovered in studies of microcephaly genes and brain size (Montgomery et al 2011), evolution of RUNX2 and face length (Pointer et al 2012) and the evolution of ASPM in both increase and decrease of brain size (Montgomery and Mundy 2012).

We studied a group of rodent species belonging to the family Cricetidae (subfamilies Arvicolinae and Cricetinae) (Lüke et al 2011). These species have a wide range of relative testes mass and therefore, inferred levels of sperm competition, as well as a diverse range of sperm head sizes. To incorporate the structural and functional differences of the two

main PRM2 domains (cleaved-PRM2 and mature-PRM2) we analyzed them separately to take into account possible differences in how they may affect sperm head size. Because sperm competition halts the relaxation of *Prm2* in this group of species (Lüke et al 2011), a negative association between the root-to-tip dN/dS ("evolutionary rate") of *Prm2* with head size was hypothesized. In addition, although PRM1 was not shown to be under postcopulatory sexual selection in these cricetid rodents, it was still expected to influence sperm head size, based on its functional similarity with PRM2 and their joint action in sperm nucleus condensation.

MATERIAL AND METHODS

Ethics

All procedures were carried out following Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65. The research protocol was approved by the Ethics Committee of the Spanish Research Council (CSIC). Wild specimens were captured with permits from the Comunidad de Madrid and the Junta de Castilla-León, Spain.

Animals

The study included 12 species of the family Cricetidae, 8 of which belong to the subfamily Arvicolinae (*Arvicola sapidus, Arvicola terrestris, Clethrionomys glareolus, Chinomys nivalis, Microtus arvalis, Microtus cabrerae, Pitymys duodecimostatus, Pitymys lusitanicus*) and 4 to the subfamily Cricetinae (*Mesocricetus auratus, Phodopus sungorus*, *Phodopus campbelli, Phodopus roborovskii)* (Steppan et al 2004). This group of species has experienced rapid evolutionary radiation and diversification (Steppan et al 2004), and shows different levels of sperm competition, as suggested by their differences in relative testes mass (Lüke et al 2011). Individuals belonging to Arvicolinae were trapped in the field during the breeding season at different locations in Spain (Gómez Montoto et al 2011b). Individuals belonging to Cricetinae stem from laboratory strains purchased from commercial suppliers and were unrelated (Lüke et al 2011). We obtained the gene sequences of at least 4 individuals per species to generate a consensus sequence. For assessments of sperm traits sample size varied between 3 and 8 individuals for each species. Males were kept in our animal facilities in individual cages, under standard

laboratory conditions in environmentally-controlled rooms (20-24ºC) on a 14 h light-10 h darkness photoperiod and provided with food and water ad libitum.

Protamine Sequences

Prm1 sequence for *Phodopus sungorus* and *Phodopus roborovskii* and *Prm1* and *Prm2* sequences for *Mesocricetus auratus* were obtained from the literature (Corzett et al 1999, Ramm et al 2008). All other nucleotide sequences were obtained through PCR amplification and sequencing.

DNA Isolation and Gene Amplification

Genomic DNA was extracted from frozen tissues using the E.Z.N.A® Tissue DNA kit (Omega, Madrid, Spain) following the manufacturer's recommendations. Protamine sequences were amplified by polymerase chain reaction (PCR). PCR mixtures were prepared in a 50 µl volume containing PCR Gold buffer 1x (Roche, Barcelona, Spain), 2.5 mM $MgCl_2$ (Roche), 0.8 mM dNTPs mix supplying 0.2 mM of each deoxinucleotide triphosphate (Applied-Biosystems, Barcelona, Spain), 0.3 mM of forward and reverse primers (Applied Biosystems), 2 U of Taq Gold DNA polymerase (Roche), and 20-100 ng/µl of genomic DNA template. All PCRs were performed in a Veriti thermocycler (Applied-Biosystems). The conditions of the thermocycler program consisted of 35-45 cycles with an initial denaturation of 95ºC for 30-40 s, an annealing stage at 52-62ºC (depending on template and primers) for 40 s, and an elongation stage at 72ºC for 30-50 s (depending on gene length). PCR primers were designed on the basis of protamine genomic sequences of other closely related rodent species accessible in the literature or in NCBI GeneBank. All alignments were performed in Bioedit (Hall 1999) and most conserved segments within untranslated regions (UTRs) were chosen. When protamines of one or more individuals of each closely related group were sequenced, new specific primers on the basis of these sequences were designed to ensure efficient PCR performance. Primer sequences can be found in Table S1. PCR products were purified by using the E.Z.N.A.® Cycle Pure kit (Omega). In cases in which additional nonspecific bands were obtained after separation in a 1.5% agarose gel, bands of about 600 bp size for *Prm2* and about 300 bp size for *Prm1* were extracted with E.Z.N.A.® Gel Extraction Kit (Omega). Purified products were sequenced (Secugen S.L., Madrid, Spain).

Alignments and Trees

Processing of the sequenced fragments was done using the sequence viewer and alignment editor BioEdit (Hall 1999). The fragments were reduced to a consensus sequence and trimmed to coding sequence. These sequences combined with database sequences were aligned on the basis of their amino acid sequences and retranslated using ClustalW implemented in BioEdit (Hall 1999). As well as an input alignment we produced an input tree to calculate the sequence evolution of *Prm1* and both domains of *Prm2* for Cricetidae. *Mus musculus* was used as outgroup. The phylogenetic tree was built based on information gathered from the literature (Jaarola et al 2004, Steppan et al 2004, Galewski et al 2006, Neumann et al 2006, Martin-Coello et al 2009, Gómez Montoto et al 2011b) (ANNEX I Fig. S1).

Evolutionary Rates (root-to-tip dN/dS)

The nonsynonymous / synonymous substitutions ratio (dN/dS) is an indicator of selective pressure at the protein level, with dN/dS = 1 indicating neutral evolution, dN/dS < 1 purifying selection, and dN/dS > 1 diversifying positive selection (Goldman and Yang 1994). To estimate rates of sequence evolution we used the application Codeml implemented in PAML 4 (Yang and Rannala 1997, Yang 2007) through the ETE toolkit (Huerta-Cepas et al 2010). The dN/dS-value was generated based on the input tree and input alignment. To obtain species-specific dN/dS values to analyze the relationship between evolutionary rate and sperm head size for each species, we used the free branch model (PAML 4, Codeml) and calculated an dN/dS value for each species by addition of dN values and dS values from the root to the terminal species branch and taking the ratio of the sum to obtain the “root-to-tip dN/dS” value.

The association between morphometric and genetic data demands the calculation of evolutionary rates that take into account not only selective pressure acting on the terminal branch (i.e., classic free branch model, Codeml, PaML4) but the accumulated selective pressure on the sequence during its evolution to the tip of the branch (root-to-tip dN/dS) in the selected group of taxa. Calculating an evolutionary rate in this way, values obtained become more comparable with measured phenotypical data since the latter also represent the accumulated evolution rather than being the result of changes solely on the terminal branch (Lüke et al 2011, Montgomery et al 2011).

Relative Testes Mass and Sperm Measurements

Animals were sacrificed by cervical dislocation, weighed and dissected immediately to remove and weigh both testes. Relative testes mass was calculated based on the rodent power function describe previously (Kenagy and Trombulak 1986) and used as in our previous study (Lüke et al 2011). Mature sperm were collected from both epididymides and vasa deferentia as described (Gómez Montoto et al 2011b) and suspended in a Hepes-buffered modified Tyrode's medium (mT-H) under air (Shi and Roldan 1995). Sperm dimensions were measured in sperm smears stained first with eosin-nigrosin and subsequently with Giemsa as described previously (Gómez Montoto et al 2011b). Spermatozoa were examined at 1000x under bright field and 200 sperm cells per male were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Phylogenetic Generalized Least Squares (PGLS) Analysis

Associations between genetic and morphometric traits should also take into account that such traits are not independent from their phylogenetic history. The phylogenetic generalized least squares (PGLS) approach (Pagel 1999) has been shown to be a powerful tool to detect associations of this kind (Rholf 2001), and it has been used in earlier studies in combination with the root-to tip dN/dS method showing genetic-morphometric associations (Lüke et al 2011, Montgomery and Mundy 2012, Pointer et al 2012, Montgimery et al 2012). We performed the PGLS analyses using the program COMPARE 4.6b (Martins 2004).

RESULTS

Sperm measurements and relative testes mass

Sperm head dimensions were analyzed in 12 species of the family Cricetidae. Measurements showed that in these species head length (HL) ranged from 4.75 µm to 8.59 µm (mean ± SEM = 6.94 ± 1.06 µm), head width (HW) varied from 2.86 µm to 4.66 µm (mean ± SEM = 3.44 ± 0.58 µm) and head elongation (HL/HW ratio) ranged from 1.54 to 2.99 (mean ± SEM = 2.06 ± 0.43). Total sperm length was also measured and it ranged from 62.69 µm to 189.26 µm (mean ± SEM = 111.30 ± 34.01 µm). Because total sperm length varies greatly among these species, and drag resulting from head size should be analyzed taking into account the length of the flagellum (Humphries et al 2008), relative

head length and width were each calculated as percentages of total sperm length (herafter "relative head length" and "relative head width“). Calculated values ranged from 4.53% to 8.02% (mean ± SEM = 6.52 ± 0.34%) for relative head length and 1.51% to 4.94% (mean ± SEM = 3.37± 0.32%) for relative head width. Relative testes mass, which is used as a proxy of sperm competition levels, varied from 0.18 to 3.61 (mean ± SEM= 1.30 ± 1.10).

Relative testes mass and sperm head size

To examine possible links between sperm competition on sperm head size in this dataset, we correlated species relative testes mass with sperm head measurements. We corrected for phylogenetic effects using the PGLS tool of the program COMPARE 4.6b and a phylogenetic tree reconstructed based on information gathered from the literature (Fig. S1). The relative testes mass of cricetid species showed significant negative relationships with relative head length (α = 15.5, CI 95% (PGLS slope) = -1.02 to -0.12, correlation = -0.62) and relative head width (α = 15.5, CI 95% (PGLS slope) = -1.10 to -0.23, correlation = -0.68) (Fig. 1A,B, Table 1). In addition, it showed a significant positive relationship with head elongation (HL/HW) (α = 15.5, CI 95% (PGLS slope) = 0.78 to 2.98, correlation = 0.72) (Fig. 4.1C, Table 4.1). There were no significant relationships between relative testes mass and uncorrected head length and head width (ANNEX I Table S2).

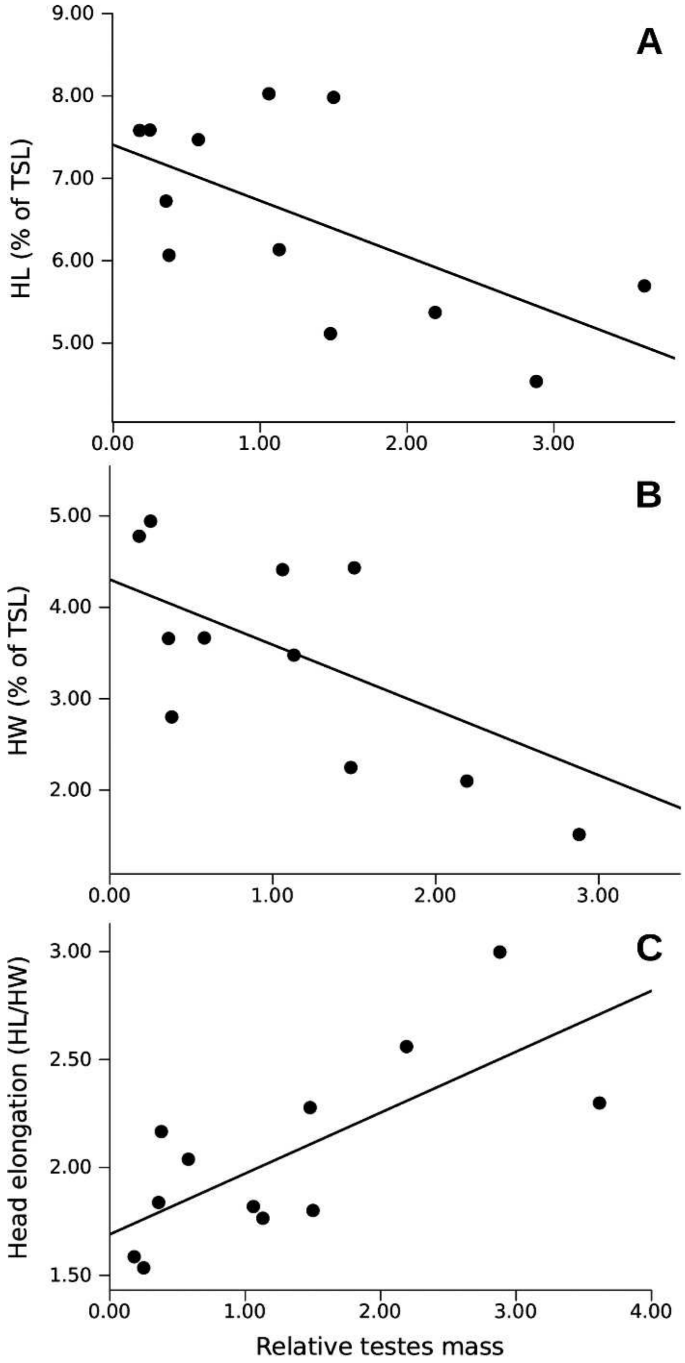


Figure 4.1. Scatterplots of significant PGLS relationships between head measurements and relative testes mass. Scatterplots shown for relationships between (A) relative sperm head length and relative testes mass, (B) relative sperm head width and relative testes mass and (C) head elongation and relative testes mass. HL = head length, HW = head width, TSL = total sperm length. Regression lines represent uncorrected least square slopes. (A) slope = -0.68, R^2 = 0.39; (B) slope = -0.71, R^2 = 0.48; (C) slope = 0.28, R^2 = 0.53.

Table 4.1. Relationships of cricetid relative testes mass with head size. CI- and CI+ indicate the confidence intervals for the PGLS regression slope (bold CI values indicate statistical significance) after PGLS analyses. lnL is the maximum likelihood estimate of alpha. Alpha is the measure of evolutionary constraints acting on phenotypes. Analyses were carried out using COMPARE 4.6b. Head length and head width are presented as percentages of total sperm length. HL/HW is the ratio between head length and head width, which is an indicator of head elongation.

	% Head length	% Head width	HL/HW
CI-	**-1.02**	**-1.10**	**0.78**
CI+	**-0.12**	**-0.23**	**2.98**
lnL	-3.82	-2.87	-2.22
alpha	15.50	15.50	15.50
corr	-0.62	-0.68	0.72

Evolutionary rate of divergence (root-to-tip dN/dS) and sperm head size

Root-to-tip dN/dS values of branch analysis (Codeml, PAML4) ranged from 0.12 to 1.17 (mean = 0.58 ± 0.30) for *Prm1*, from 0.19 to 0.51 (mean = 0.33 ± 0.096) for cleaved-*Prm2* and from 0.28 to 1.66 (mean = 1.00 ± 0.55) for mature-*Prm2.* To test the relationship of protamine evolution with sperm head measurements we correlated the species dN/dS value calculated from the root with the species sperm head measurements. We corrected for phylogenetic effects using the PGLS tool of the program COMPARE 4.6b.

Prm1 root-to-tip dN/dS values for the different species showed significant positive relationships with both relative head length (α = 8.82, CI 95% (PGLS slope) = 0.16 to 0.29, correlation = 0.90) and relative head width (α = 8.59, CI 95% (PGLS slope) = 0.16 to 0.31, correlation = 0.88) (Fig. 4.2A,B, Table 4.2). On the other hand, *Prm1* root-to-tip dN/dS values showed a significant negative relationship with head elongation (HL/HW) (α = 2.78, CI 95% (PGLS slope) = -0.73 to -0.14, correlation = -0.67) (Fig. 4.2C, Table 4.2). There was a significant relationship between the evolutionary rate of *Prm1* and uncorrected head width, but not with uncorrected head length (ANNEX I Table S3).

Cleaved-*Prm2* root-to-tip dN/dS values showed significant positive relationships with relative head length (α = 7.19, CI 95% (PGLS slope) = 0.03 to 0.10, correlation = 0.77) and with relative head width (α = 7.64, CI 95% (PGLS slope) = 0.03 to 0.11, correlation = 0.76) (Fig. 4.2D,E, Table 4.2). Furthermore, cleaved-*Prm2* root-to-tip dN/dS values had a significant negative relationship with head elongation (HL/HW) (α = 7.99, CI 95% (PGLS slope) = -0.28 to -0.08, correlation = -0.74) (Fig. 4.2F, Table 4.2). In contrast, mature-*Prm2* exhibited no significant relationship between root-to-tip dN/dS values and head measurements (Table 4.2). Relationships with uncorrected head length and head width were non significant for cleaved-*Prm2* and mature-*Prm2* (ANNEX I Table S3).

Table 4.2. Relationships of protamine evolutionary rates with head size. CI- and CI+ indicate the confidence intervals for the PGLS regression slope (bold CI values indicate statistical significance) after PGLS analyses, lnL the maximum likelihood estimate of alpha, and alpha the measure of evolutionary constraints acting on phenotypes (COMPARE 4.6b). Head length and head width are presented as percentages of total sperm length. HL/HW is the ratio between head length and head width, which is an indicator of head elongation.

		% Head length	% Head width	HL/HW
Protamine 1	CI-	**0.16**	**0.16**	**-0.73**
	CI+	**0.29**	**0.31**	**-0.14**
	lnL	19.44	18.54	15.03
	alpha	8.82	8.59	2.78
	corr	0.90	0.88	-0.67
cleaved *Protamine 2*	CI-	**0.03**	**0.03**	**-0.28**
	CI+	**0.10**	**0.11**	**-0.08**
	lnL	27.48	27.40	26.88
	alpha	7.19	7.64	7.99
	corr	0.77	0.76	-0.74
mature *Protamine 2*	CI-	-0.02	-0.06	-0.70
	CI+	0.41	0.39	0.43
	lnL	8.19	7.82	7.11
	alpha	1.32	1.07	0.59
	corr	0.49	0.40	-0.15

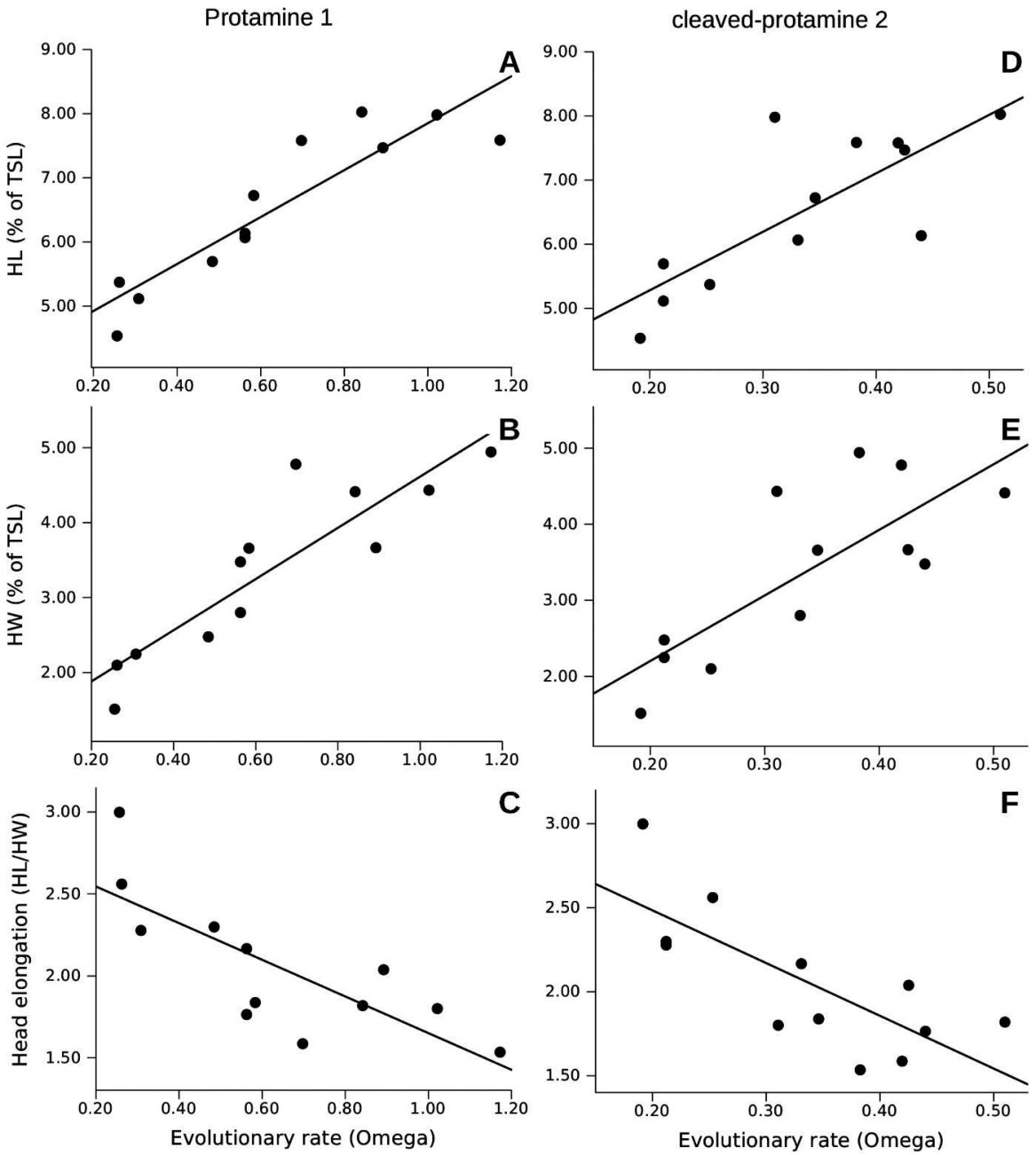


Figure 4.2. Scatterplots of significant PGLS relationships between head measurements and protamine evolutionary rates (root-to-tip dN/dS). Scatterplots shown for relationships between (A) relative sperm head length and evolutionary rate of protamine 1, (B) relative sperm head width and evolutionary rate of protamine 1, (C) head elongation and evolutionary rate of protamine 1 and (D) relative sperm head length and evolutionary rate of cleaved protamine 2, (E) relative sperm head width and evolutionary rate of cleaved protamine 2, (F) head elongation and evolutionary rate of cleaved protamine 2. HL = head length, HW = head width, TSL = total sperm length. Regression lines represent uncorrected least square slopes. (A) slope = 3.65, R^2 = 0.82; (B) slope = 3.41, R^2 = 0.79; (C) slope = -1.12, R^2 = 0.60; (D) slope = 9.12, R^2 = 0.61; (E) slope = 8.62, R^2 = 0.61; (F) slope = -3.14, R^2 = 0.57.

DISCUSSION

In this study we examined genetic-morphometric associations by analyzing differences in protamine gene sequences and sperm head size and elongation, with the aim of understanding the role of sperm competition on reproductive trait evolution. Earlier studies have successfully used this approach to assess genotype-phenotype associations in brain size and face length (Montgomery et al 2011, Pointer et al 2012, Montgomery and Mundy 2012) and, to the best of our knowledge, this is the first study to explore this association in germ cells. We found significant associations between the evolutionary rates (root-to-tip dN/dS values) of *Prm1* and cleaved-*Prm2* gene sequences and sperm head morphometry, while no significant relation was found for the mature domain of *Prm2*. The evolutionary rates of both *Prm1* and the cleaved domain of *Prm2* show positive relations with relative head length and relative head width, and negative relations with head elongation (i.e., HL/HW ratio). These parameters of sperm morphometry are also significantly associated with the species relative testes mass, which represent inferred levels of sperm competition (Soulsbury 2010). Thus, these results report for the first time evidence of a relationship between differences in protamine coding sequences and sperm head shaping.

An analysis of protamine promoter sequences in mouse species found that divergence of *Prm2* promoters (which may result in differences in protamine expression) is positively correlated with sperm velocity (Martin-Coello et al 2009). Such study suggested a possible effect of protamines on head shape and, hence, on the sperm hydrodynamic efficiency which, in consequence, may influence sperm velocity. A direct relationship between sequence divergence and sperm head shape or size in this group of closely related mouse species was not apparent (Martin-Coello et al 2009) perhaps because subtle changes in sperm head phenotype could not be identified with the methods employed in that study.

We found positive relationships between *Prm1* and cleaved-*Prm2* divergence and relative head length and width in cricetid rodents, a group that started diverging 16-18 million years ago (Steppan et al 2004), in contrast to a more recent emergence of mouse species which is thought to have started about 5 million years ago (Guénet and Bonhomme 2003). A higher sequence divergence for both *Prm1* and cleaved-*Prm2* correlates with longer and wider and, therefore, bigger heads in relation to total sperm length. The relationships of sperm head elongation (HL/HW) with sequence divergence were negative for both *Prm1* and cleaved-*Prm2*. A lower sequence divergence coincided with more elongated heads. Analyses of possible associations between sperm head size and elongation with relative

testes mass, which we used as inferred levels of sperm competition, revealed a different association between these parameters. Species exhibiting high relative testes mass (i.e., higher inferred sperm competition levels) showed smaller, more elongated sperm heads relative to total sperm length. This agrees with earlier comparative studies in which it was found that more elongated and smaller heads in relation to flagellum length are favoured by sperm competition in mammals (Tourmente et al 2011a).

Elongation of the sperm head as well as lower relative sperm head size are thought to reduce drag on the sperm cell, increasing its hydrodynamic efficiency and therefore its swimming speed (Malo et al 2006, Humphries et al 2008). Since swimming speed is a major factor in fertilization success (Birkhead et al 1999, Gage et al 2004) an improvement of the hydrodynamic efficiency should be strongly favoured by postcopulatory sexual selection. The correlations of evolutionary rates of *Prm1* and cleaved-*Prm2* with sperm head size and elongation, and the significant role played by head size and elongation in making sperm more competitive, support the hypothesis that protamines may influence head shape and sperm's hydrodynamic properties, and sperm competitive capacity in general.

It is noteworthy that we did not find significant correlations between sequence divergence of mature-*Prm2* and sperm head size and elongation, although there were strong relationships between cleaved-*Prm2* and sperm phenotype. Both PRM1 and mature-PRM2 appear to share the role of condensing DNA, whereas the function of cleaved-PRM2 is largely unknown. The uncleaved PRM2 precursor binds to DNA and is cleaved over a period of several days until only the mature-PRM2 is left bound to the DNA (Carre-Eusebe et al 1991, Chauvière et al 1992). Since DNA condensation has been found to coincide temporally with the start of protamine translation and posttranslational processing (Kierzenbaum and Tres 1975, Lee et al 1995, Brewer et al 2002) it is reasonable to envisage that the cleaved-PRM2 domain may have a more important function during the actual process of DNA condensation than the mature-PRM2 domain. Previous studies have focussed mainly on mature-PRM2, but our results suggest a more important role for cleaved-PRM2 in the process of DNA condensation and head shaping, thus warranting further studies of the role of cleaved-PRM2 and the evolution of this domain.

Our previous work on the same group of cricetid species found that both cleaved-*Prm2* and mature-*Prm2* are affected by relaxation (Lüke et al 2011). Sexual selection was found to halt the relaxation of the *Prm2* gene, as shown by a negative relationship between sequence divergence and relative testes mass. Furthermore, *Prm1* was found to be

functionally conserved with directed positive selection on specific functional sites, but it was not influenced by sexual selection (Lüke et al 2011). In the present study, we observed that more divergent coding sequences of *Prm1* and cleaved-*Prm2* were associated with proportionately bigger and less elongated sperm heads, traits which may be less favourable in competitive situations, while higher levels of sperm competition associated with relatively smaller and more elongated sperm heads. Our current results thus suggest possible reasons why sexual selection may act to halt relaxation in *Prm2*. Higher sequence divergence in cleaved-*Prm2* seems to be related to an enlargement and reduction of elongation of the sperm head. Thus, divergence in cleaved-*Prm2* might have a negative effect on the hydrodynamic efficiency of the sperm cell, and therefore sexual selection appears to halt relaxation in *Prm2* to preclude such decrease in hydrodynamic efficiency. Previous results showing no evidence of sexual selection on *Prm1* (Lüke et al 2011) seem to be at odds with our current results linking changes in head size and elongation with increased *Prm1* sequence divergence. However, in our previous study *Prm1* was shown to be functionally conserved allowing rapid changes in very specific functional sites. This functional conservation may be sufficient to ensure a hydrodynamically-efficient sperm head shape in competitive situations. Due to its function and efficiency in DNA condensation, *Prm1* can be expected to affect sperm head size and elongation even though it does not appear to be influenced by sexual selection.

In conclusion, this study presents the first evidence for a potential link between divergence in protamine coding sequences and sperm head size and elongation. Because a strong correlation between sperm head phenotype and the PRM2 domain that is cleaved off during sperm chromatin condensation was found, it could be argued that this cleaved-PRM2 domain may have a role in regulating the process of DNA condensation. On the other hand, the role of mature-PRM2 (i.e., the PRM2 domain remaining after cleavage and which stays attached to DNA) might be restricted to maintenance of the condensed state of DNA in the differentiated mature sperm. Lower sequence divergence in *Prm1* and cleaved-*Prm2* may be important for proportionately smaller and more elongated sperm heads, which seems to be favoured by sperm competition in this group of rodents. This evidence supports previously hypothesized involvement of protamines in sperm head shaping. Additionally the proposed negative influence of cleaved-*Prm2* divergence on sperm head shaping offers a possible explanation for sexual selection acting to halt relaxation of this sequence in rodents.

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CHAPTER 5

SEXUAL SELECTION ON PROTAMINE 1 IN MAMMALS

RESUMEN

Las protaminas tienen un papel crucial en la fertilidad masculina. Se encuentran involucradas en el empaquetamiento de la cromatina espermática e influyen en la forma de la cabeza celular y, por tanto, son importantes para la función de los espermatozoides. La estructura de las protaminas es básica, con numerosos dominios ricos en arginina que se unen al ADN. La selección sexual postcópula parece tener un papel importante en la evolución de la secuencia y expresión de las protaminas. En este estudio se analizan los patrones de evolución y selección sexual (en forma de competición espermática) que actúan sobre la secuencia génica de la protamina 1 en 237 especies de mamíferos. Se examinaron patrones en común así como diferencias entre las principales subclases de mamíferos (Euterios, Metaterios) y en diferentes clados. Se encontró que un alto contenido de arginina en la protamina 1 se asocia con una cabeza espermática menos ancha que puede ser importante para la velocidad de natación de los espermatozoides. El alto contenido de arginina en la protamina 1 en los mamíferos parece estar favorecido por la selección sexual. En metaterios, la longitud de la secuencia se encuentra favorecida por la selección sexual. Se observaron diferencias en presiones selectivas sobre secuencias y en sitios de codones entre clados de mamíferos, y se halló que la tasa evolutiva y el contenido de arginina se encuentra relacionado a la selección sexual en Rodentia y Diprodontia. Este estudio puso en evidencia un patrón evolutivo complejo de la protamina 1, que demostró tener diferentes restricciones selectivas y efectos de la selección sexual en diversos grupos de mamíferos. En contraste, el efecto del contenido de arginina sobre la forma de la cabeza espermática, y la posible participación de la competición espermática, se identificó en todos los mamíferos.

SUMMARY

Protamines have a crucial role in male fertility. They are involved in sperm chromatin packaging and influence the shape of the sperm head and, hence, are important for sperm performance. Protamine structure is basic with numerous arginine-rich DNA-binding domains. Postcopulatory sexual selection is thought to play an important role in protamine sequence evolution and expression. Here we analyze patterns of evolution and sexual selection (in the form of sperm competiton) acting on protamine 1 gene sequence in 237 mammalian species. We assessed common patterns as well as differences between the major mammalian subclasses (Eutheria, Metatheria) and clades. We found that a high arginine content in protamine 1 associates with a lower sperm head width, which may have an impact on sperm swimming velocity. High arginine content in protamine 1 across mammals appears to be promoted by sexual selection. In metatherians, sequence length was found to be sexually selected. Differences in selective pressures on sequences and codon sites were observed between mammalian clades, and evolutionary rate and arginine content was related to sexual selection in Rodentia and Diprotodontia. Our study revealed a complex evolutionary pattern of protamine 1, with different selective constraints, and effects of sexual selection, between mammalian groups. In contrast, the effect of arginine content on head shape, and the possible involvement of sperm competition, was identified across all mammals.

INTRODUCTION

Understanding how evolutionary processes generate the large variation observed among species is one of the fundamental questions in evolutionary biology. Sperm competition, as a form of postcopulatory sexual selection, is a unique evolutionary process which acts on sperm of males competing for the fertilization of ova (Parker 1970). This selective process drives the adaptation of sperm form, function and fertility (reviewed in Birkhead and Moller 1998, Simmons 2001, Birkhead et al 2009). Uncovering how sperm competition can shape phenotypes through changes in gene and regulatory DNA sequences would allow us to draw conclusions about evolutionary process in general, the evolution of function, and causes underlying medical conditions such as sub- or infertility.

Sperm morphologies, especially the sperm head, are highy variable among species (Cummins and Woodall 1985, Roldan et al 1992, Pitnick et al 2009). Changes in sperm head size and shape are important determinants of sperm swimming velocity that, in turn, is a key determinant of male fertility. Sperm head morphology seems to be greatly influenced by the condensation of nuclear chromatin (Balhorn 2007, Cree et al 2011). Protamines, which are basic arginine-rich sperm nuclear proteins, have an important role in sperm chromatin condensation. They are involved in a train of successive replacements of histones to transition proteins to protamines (Oliva and Dixon 1991). This process results in a tightly packed, efficiently shielded chromatin and an almost complete silencing of expression (Balhorn 2007). Due to the existence of cysteine residues, protamines of eutherian mammals form disulphide bonds within and between protamines stabilizing the chromatin structure (Balhorn et al 1992, Queralt et al 1993).

Because protamines are crucial for the process of chromatin condensation, alterations in their expression can affect fertility (Cho et al 2003, Aoki et al 2005, Oliva 2006, Carrel et al 2007). In men, changes in sperm protamine content affects sperm head morphology, as well as sperm motility (Aoki et al 2005). In mouse models, modification of the protamine content is linked to sperm morphological abnormalities and decreases in sperm motility (Cho et al 2001). Furthermore, changes in protamine gene sequences and protamine expression ratios affect head size and shape in rodents (Lüke et al 2014a,b).

Protamines can be found in the sperm of protostomes as well as deuterostomes (Oliva and Dixon 1991). They most likely evolved in chordates from a sperm-specific histone H1 through a shift from a lysine-rich histone H1 to the arginine-rich protamine (Lewis et al 2004). The selection for an arginine-rich protamine is thought to be driven by selective

constraints imposed by internal fertilization (Kansinsky et al 2011). Arginine richness, as opposed to lysine, results in a higher affinity for the protein to bind to the DNA molecule as well as a greater binding flexibilty due to its guanidinium group (Ausio et al 1984, Cheng et al 2003). In mammals, two types of protamines have been identified. Protamine 1 is found in all mammals and shows sequence similarities to bird protamine 1 (Oliva and Dixon 1991). Protamine 2 is found in primates and rodents but evidence for the existence of the protamine 2 gene sequence, transcripts and, in some cases, mature protein has been presented for several species of other mammalian clades (Oliva 2006, Balhorn 2007). Mammalian protamines contain three or more DNA anchoring domains which comprise 3-7 arginine residues that are separated by uncharged amino acids (Balhorn et al 1999). Within mammals in general protamines are thought to be diverse, especially in the C-terminal region, but contain conserved regions that are also found in birds (N-terminal ARYR, SRSRSR phosphorylation site, 3 arginine clusters) (Queralt et al 1993). In marsupials (and birds) cysteine residues are absent except in the *Planingale* genus.

Several studies claim that protamines are the fastest evolving reproductive proteins, exhibiting high structural heterogenity, when compared to other sperm nuclear basic proteins, (Oliva and Dixon 1991, Wyckoff et al 2000). However, evidence of purifying selection acting on the maintenance of the high arginine concentration exists, while the position of arginine residues seems to be variable, leading to the conclusion that protamine 1 may be affected by positive and purifying selection alike (Rooney et al 2000). Evidence of positive selection in the protamine 1 gene sequence has been detected in a small group of primates (Rooney et al 1999, Wyckoff et al 2000). Other studies have demonstrated different selective constraints in other mammalian species (Martin-Coello et al 2009, Lüke et al 2011). Among cricetid rodents we showed that protamine 1 is under conserved selective constraint with signs of positive selection restricted to specific codon sites. On the other hand, the gene sequence of protamine 2 was shown to be under relaxed constraint on the way to degradation.

There is limited evidence for an effect of sperm competition on coding sequences of sperm proteins. The seminal fluid proteins SEMG2 and SVS, the sperm surface proteins ADAMs 2 and 18, and the acrosomal proteins Zonadhesin and SPAM1 have all been found to be positively affected by level of sperm competition in primates (Dorus et al 2004, Herlyn and Zischler 2007, Ramm et al 2008, Finn and Civetta 2010, Prothmann et al 2012). A negative effect of sperm competition on coding sequences of seminal fluid proteins in butterflies (Walters and Harrison 2011), and on protamine 1 and protamine 2 in rodents has been

reported (Lüke et al 2011). The evolution of protamine genes and regulatory sequences, as well as their expression, is affected by sperm competition in different groups of rodents. Contrary to expectations, sexual selection could not be detected to act on protamine 1 gene in cricetids or murids. In contrast, sperm competition was shown to reduce the relaxation acting on the gene sequence of protamine 2, resulting in a more conserved state of the gene in species with high levels of sperm competition (Lüke et al 2011).

Until now sexual selection on protamine gene sequences and its effect on sperm head phenotype has only been studied in rodents. Thus, in this study we aimed to analyze evolutionary patterns of protamine 1 coding sequence and their effects on sperm head dimensions on a broader scale across mammals. The extensive availabilty of data on protamine 1 sequence in Genbank allowed us to study the evolution and selection of protamine 1 across major mammalian clades. Here we analyze the evolution and selection of protamine 1 in 237 mammal species. We examined the evolutionary rate on whole sequence and site level and tested for sexual selection and possible effect on head dimensions. Additionally, we tested for sexual selection acting on arginine content. We expected to find differences in selective constraints between clades especially in clades with high diversity in levels of sperm competition. We hypothesized that protamine 1 would be sexually selected across mammals and that changes in protamine 1 coding sequence and arginine content may influence head dimensions.

MATERIAL AND METHODS

Sequence data and phylogenetic tree

Gene sequences of mammalian protamine 1 for which at least 10 species were available were obtained from NCBI Genbank and previous publications (Lüke et al 2011) (ANNEX II Table S3), visualized with Geneious 5.5.9 (Biomatters, http://www.geneious.com/) and trimmed to coding sequence based on NCBI Genbank information. Sequences were manually checked to ensure correct trimming. Translation alignments based on the muscle alignment algorithm implemented in Geneious 5.5.9 were performed and checked manually. In addition to an aligment including all 237 mammalian species (translated alignment see Fig. S5) we performed separate alignments for each mammalian clade studied (Primates, Rodentia, Chiroptera, Cetacea, Artiodactyla, Dasyuromorpha,

Diprotodontia) as well as separate alignments for eutherians and metatherians (translated alignment see Figs S6 & S7). Amino acid frequencies, pairwise percent identity and percentage of identical sites were calculated using Geneious 5.5.9 for each alignment (ANNEX II Table S1).

The phylogenetic tree of 237 included mammalian species was constructed as a consensus of phylogenies available in literature (ANNEX II Fig. S8 and references therein).

Phenotype data

Data on body mass, testes mass and sperm dimensions were obtained from the literature (ANNEX II Table S3 and references therein). Testes and body mass data were available for 132 of the 237 species for which protamine 1 information was available. Data on sperm head width were found for 65 species, and sperm head length for 87 species. Residual testes mass data were obtained from a regression analysis including body mass as independent and testes mass as dependent variable. Residual testes mass was obtained for each clade separately due to previously reported differences of body mass and testes mass regression slopes between clades (MacLeod 2010). Residual testes mass was used in this study to assign species to sperm competition groups and for graphical representation of multiple regression results. Because total sperm length varies greatly among these species, and drag resulting from head size should be analyzed taking into account the length of the flagellum (Humphries et al 2008), sperm head length and head width were each employed in analyses as proportions of total sperm length (TSL) (hereafter, relative HL and relative HW).

Analysis of selective pressures

The nonsynonymous/synonymous substitutions rate ratio ($\omega = dN/dS$) is an indicator of selective pressure at the protein level, with $\omega=1$ indicating neutral evolution, $\omega<1$ purifying selection, and $\omega>1$ diversifying positive selection (Goldman and Yang 1994). To estimate rates of sequence evolution we used the application Codeml implemented in PAML 4 (Yang and Rannala 1997, Yang 2007). Applied models are explained in Supplementary materials and methods. Likelihood-ratio-tests (LRT) were performed to test if the alternative model presents a better fit to the dataset against the null model. For the Codeml codon frequency setting, as well as the setting for number of categories, we used the setting with the best fit for each analysis according to the preliminary likelihood-ratio-

analysis. Branch lengths calculated in the model M0 “one-ratio” (see Supplementary materials and methods) were used as input for subsequent models.

Phyologenetically corrected regression analysis (PGLS)

To test for correlations between sequence evolution and a proxy of sperm competition levels, or head dimensions, we employed the phylogenetic generalized least squares approach (PGLS) (Freckleton 2002). Body mass and testes mass, as a proxy for sperm competition levels ("relative testes mass") were included as independent variables in a multiple PGLS regression. Associations between genetic and morphometric traits should also take into account that such traits are not independent from their phylogenetic history (Harvey and Pagel 1991). The PGLS approach has been shown to be a powerful tool to detect associations of this kind (Rholf 2001), and it has been used in earlier studies in combination with the root-to tip dN/dS method showing genetic-morphometric associations (Lüke et al 2011, Montgomery et al 2011, 2012, Pointer et al 2012). We performed PGLS analysis using the R-package caper.

RESULTS

Sequence properties

Amino acid frequency and sequence similarity within mammals, as well as within eutherians and marsupials, and within different clades, were compared. Results are shown in Table S1. Metatheria showed significantly higher relative arginine content than Eutheria ($t(219.33)=-17.26$, $p<0.001$). Within Eutheria, a one-way ANOVA revealed signifcant differences in arginine content between clades ($F(4,144)=100$, $p<0.001$). Post-hoc comparisons showed that Chiroptera and Rodentia exhibit significantly higher content of arginine in the coding sequence of protamine 1 (Fig. 5.1A, ANNEX II Table S2). Within Metatheria, Diprotodontia showed a signifcantly higher arginine content than Dasyuromorpha ($t(52.8) = 9.42$, $p < 0.001$)(Fig. 5.1B).

Measures of sequence divergence and amino acid frequency within clades are shown in Table S1 (ANNEX II). Considering the differences in sequence properties between Eutheria and Metatheria, as well as the early separation of these two groups, all subsequent analyses within clades were also done separatedly for eutherian and metatherian clades.

Selective pressures across mammals

We tested for the general mode of selection acting on protamine 1 in mammals. To obtain the backround pressure acting on the whole sequence across all mammals we calculated the evolutionary rate (ω) for the whole tree on the whole protamine 1 sequence (Codeml (PAML4) model M0 as explained in Supplementary materials and methods). The evolutionary rate calculated across mammals in model M0 was ω=0.38.

Comparison of selective pressures

To assess the comparative selective pressures for the entire protamine 1 sequence and the directed selective pressures on codon sites we employed the branch analysis and the branch-site analysis (see Supplementary materials and methods), alternatively marking metatherians as foreground against eutherians as background, and then eutherians as a foreground against metatherians as background. The branch analysis comparing Eutheria and Metatheria suggests a significantly stronger selective constraint on metatherians than eutherians (Eutheria: LRT M0 vs MC non significant, M0 ω considered, Metatheria: LRT M0 vs MC significant, MC ω considered). In any case, both clades seem to be evolving under purifying selection (MCfixed vs MC significant, ω is significantly lower than 1) (Table 5.1). The branch-site test showed no directed selection on codon sites for Eutheria or Metatheria (BSfixed vs BS non significant)(Table 5.1).

Secondly, we tested for differences between mammalian clades by employing branch- and branch-site analyses for each group, marking the clade under analysis as foreground against their corresponding eutherian or metatherian background. In the comparison of eutherian clades, we found that Primates, Rodentia and Cetacea showed selective constraints different from the background (M0 vs MC significant, MC ω considered). In contrast, Chiroptera and Artiodactyla showed similar selective constraint as their background (M0 vs MC non significant, M0 ω considered). Primates and Cetacea showed signs of relaxed selective constraint (MCfixed vs MC non significant, ω did not differ significantly from 1), while Rodentia, Chiroptera and Artiodactyla showed evidence for purifying selection (MCfixed vs MC significant, ω is significantly lower than 1). Branch-site analysis revealed evidence for positive selection on codon sites for Chiroptera and Cetacea (BSfixed vs BS significant, PSS detected). For Artiodactyla Bsfixed vs BS is significant while no positively selected sites were detected. This might be interpreted as evidence for high purifying selection on codon sites. Primates and Rodentia showed no evidence for positive selection at the site level (BSfixed vs BS non significant) (see Table

5.1).

In the comparison of metatherian clades neither Dasyuromorpha nor Diprotodontia showed selective constraints differing from the background. The selective constraint is therefore considered the same for both groups (M0 vs MC non significant, M0 ω considered). Diprotodontia showed evidence of purifying selection acting on the protamine 1 coding sequence (MCfixed vs MC significant, ω is significantly lower than 1) while the likelihood ratio test of MCfixed vs MC was not significant for Dasyuromorpha, likelihood ratio test M0 vs MC shows this clade to be evolving under the same selective constraints as the background and Diprotodontia. We therefore consider purifying selection to be the most probable mode of selection acting on this clade. The branch-site test showed directed positive selection on codon sites for Dasyuromorpha and Diprotontia (BSfixed vs BS significant)(Table 5.1).

The root-to-tip ω calculated for eutherian mammals is shown in Table S3 (ANNEX II).

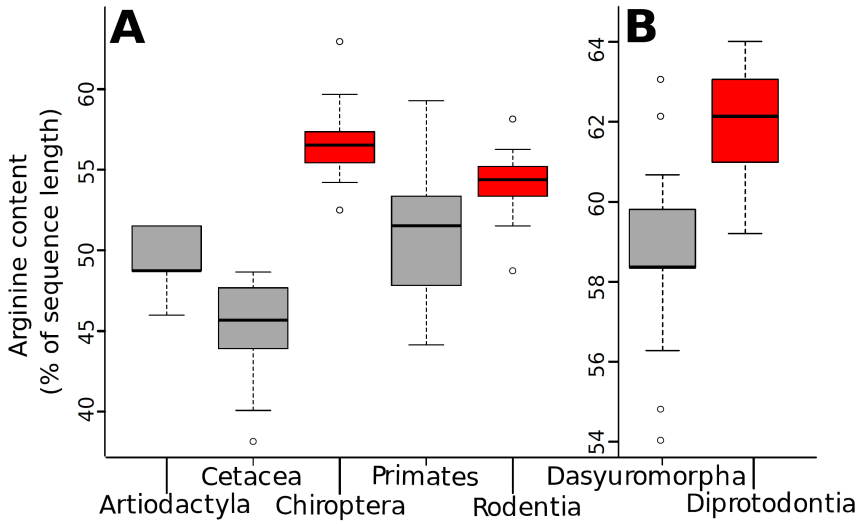

Figure 5.1. Boxplots for arginine content (as percentage of total sequence length). (A) Eutherian clades. (B) Metatherian clades. Clades with comparatively high arginine content within each subclass are highlighted in red. Comparisons are statistically significant between all clades except between Artiodactyla and Primates.

Sexual selection

Rationale for analyses of possible effects of sexual selection

To examine the possible effects of sexual selection on protamine 1 coding sequence within all mammals and, subsequently, for each clade, we chose two approaches. For the first approach (Test 1) we divided taxa into three sperm competition groups (high, intermediate and low levels) according to the species levels of sperm competition inferred from their residual testes mass. To define the three groups (high, intermediate, low) we first split the data into two groups (high, low) with the average as point of division and subsequently defined an intermediate group in which we included data points that fell below the average of the “high” group and above the average of the “low” group. For each sperm competition group we performed a branch analysis (see Supplementary materials and methods) to detect differences in selective pressures acting on the whole sequence. Subsequently, to assess sexual selection within clades we ran branch analyses of sperm competition groups within clades.

As a second approach (Test 2) we used a phylogenetic generalized least squares (PGLS) regression analysis within mammals and clades (see below). The root-to-tip ω (see Supplementary materials and methods) as well as the arginine content (as percent of sequence length) were included as dependent variables. Body mass and testes mass were used as independent variables ("relative testes mass", a well recognized proxy for sperm competition; Birkhead et al 2009, Gomendio et al 1998, Soulsbury and Dornhaus 2010). We tested for a relationship between evolutionary rate (root-to-tip ω) on arginine content by including the latter as dependent variable and the former as independent variable in the PGLS regression. Furthermore we calculated the clades' median absolute deviation of residual testes mass and arginine content to test for an effect of variability of sperm competition ("median absolute deviation of residual testes mass") on the clades' ω value (M3, see Supplementary materials and methods), arginine content, and arginine content variability ("median absolute deviation of arginine content"). The median absolute deviation (or absolute deviation around the median) is a robust measure of variability. It is calculated as the median of the absolute deviations from the data's median (i.e., the median of each absolute value minus the median) and it is not sensitive to the presence of outliers (Huber 1981).

Sexual selection across mammals

Test 1 of sexual selection (branch analysis of three sperm competition groups) showed no differential selective constraint for any sperm competition group within all mammals (M0 vs

MC non significant, M0 ω considered) although MC reports a comparably lower ω for high sperm competition species (Table 2). Test 2 of sexual selection (PGLS regession of root-to-tip ω with sperm competition proxy) showed no significant correlations with residual testes mass for protamine 1 root-to-tip ω in mammals. A nearly significant positive correlation was found for arginine content with relative testes mass (Fig. 5.2A, ANNEX II Table S4). Arginine content was significantly negatively correlated with root-to-tip ω (ANNEX II Table S4).

Sexual selection within clades

Test 1 of sexual selection (branch analysis for three sperm competition groups) showed, in Rodentia, differential selective constraint for species with low residual testes size (M0 vs MC significant, MC ω considered), with evidence of relaxed selective constraint in the low residual testes mass group (MCfixed vs MC non significant, ω not significantly different from 1). Groups with intermediate and high residual testes mass showed evidence of purifying selection (MCfixed vs MC significant, ω significantly lower than 1) (Table 2). For Diprotodontia, test 1 of sexual selection showed differential selective constraint for species with high residual testes masses (M0 vs MC significant, MC ω considered), showing evidence for strong purifying selection acting on the high residual testes mass group. In the intermediate and low residual testes mass groups comparatively weaker purifying selection was detected (MCfixed vs MC significant, ω significantly lower than 1) (Table 5.2). For all other clades, the likelihood ratio tests for test 1 (M0 versus MC) were not significant suggesting that no specific selection is occuring in species with high levels of residual testes mass (Table 5.2).

Results obtained using test 2 of sexual selection (PGLS regression between root-to-tip ω and sperm competition levels) supported the results gathered using test 1. PGLS regessions showed significant negative correlations between root-to-tip ω and corrected testes mass for Rodentia and Diprotodontia (Fig. 5.2C, ANNEX II Table S4). Additionally, we observed significantly positive correlations between corrected testes mass and arginine content (as percent of sequence length) in the protamine 1 amino acid sequences of Rodentia and Diprotodontia (Fig. 5.2A, ANNEX II Table S4). We found that protamine 1 evolutionary rates correlated negatively with arginine content in Primates, Artiodactyla, Rodentia and, to a lesser extent, in Dasyuromorpha. A trend for this relationship was found in Chrioptera and Diprotodontia, while no relationship was found in Cetacea (ANNEX II Table S4). Tests for sexual selection could not be done for Artiodactyla or Chiroptera due to limited data for testes mass.

Median absolute deviation of relative testes mass for clades correlated positively with the clade's ω (M3) and with the clade's median absolute deviation of arginine frequency. On the other hand, it correlated negatively with the clade's mean arginine frequency (ANNEX II Table S2 and S4).

Because the sequence of protamine 1 was significantly longer in marsupials than in eutherians ($t(227.5) = -72.69$, $p < 0.001$) (Fig. 5.3A) we tested for possible relationships between sexual selection and sequence length within this subclass. We found a significant positive correlation between relative testes mass and sequence length in Metatheria and in Diprotodontia (Fig. 5.3B, ANNEX II Table S4).

Table 5.1. Results of branch analyses and branch-site analyses for eutherian and metatherian clades, as well as Eutheria and Metatheria.

	LRTs for branch analysis				LRTs for branch-site analysis		Proportion of sites in ω site classes (BS):						interpretation	
Foreground branches	2Δ (M0-MC)	*P*	2Δ (MCfixed-MC)	*P*	2Δ (BSfixed-BS)	*P*	ω	0	1	2a	2b	Positively selected sites (BEB p<0.05)	Selection over whole seqence	Directed selection on sites
Primates	13.77	0.01	1.09	ns	3.02	ns	0.791	0.53	0.15	0.25	0.07		relaxed	non significant PSS
Rodentia	12.38	0.01	71.95	0.00	2.12	ns	0.226	0.65	0.32	0.02	0.01		purifying	no signal
Chiroptera	-0.15	ns	30.26	0.00	15.34	0.00	0.385	0.65	0.27	0.06	0.02	54-, 55Y	puriyfing	positive
Artiodactyla	0.28	ns	6.15	0.05	5.48	0.05		0.68	0.32	0.00	0.00		puriyfing	purified
Cetacea	15.57	0.00	-5.70	ns	5.65	0.05	0.94	0.43	0.20	0.26	0.12	5R, 15C, 28C, 32R, 35R, 55Y, 60R	relaxed	positive
Dasyuromorphia	-10.37	ns	2.19	ns	18.05	0.00	0.237	0.73	0.24	0.02	0.01	27V, 28R	purifying	positive
Diprotodontia	2.57	ns	100.76	0.00	23.16	0.00	0.237	0.74	0.12	0.12	0.02	41R, 42R, 43R, 45R, 47K, 48G	purifying	positive
Eutheria	2.71	ns	134.76	0.00	-2.80	ns	0.376	0.44	0.28	0.17	0.11		purifying	non significant PSS
Metatheria	5.67	0.05	146.75	0.00	-3.87	ns	0.273	0.57	0.26	0.12	0.05		purifying	non significant PSS

LRT= Likelihood ratio test, ω=clade's ω as calculated by branch analysis; if LRT of M0 versus MC significant MC, ω is reported; if LRT is non significant, M0 ω is reported. PSS=positively selected sites. ω site classes: 0: 0<ω<1 for foreground and background branches, 1: ω=1 for foreground and background branches, 2a: 0<ω<1 for background and ω>1 for foreground, 2b: ω=1 for background and ω>1 for foreground.

Table 5.2. Summary of branch analyses within clades, with species grouped by level of sperm competition.

Clade	Sperm competition group	LRT M02Δ (M0-MC)	*P*	2Δ (MCfixed-MC)	*P*	ω group specific?	ω different from 1?	ω (MC)	ω (M0)	interpretation
Primates	low	0.03	ns	0.07	ns	no	no	1.198		relaxed
	intermediate	1.67	ns	1.61	ns	no	no	2.446	1.074	relaxed
	high	0.07	ns	0.02	ns	no	no	0.898		relaxed
Rodentia	low	5.42	0.05	0.47	ns	yes	no	0.680		relaxed
	intermediate	0.00	ns	18.92	0.00	no	yes	0.210	0.214	purified
	high	2.10	ns	23.15	0.00	no	yes	0.117		purified
Cetacea	low	0.02	ns	0.03	ns	no	no	1.20		purified/relaxed
	intermediate	1.69	ns	1.30	ns	no	no	1.84	1.037	purified/relaxed
	high	0.44	ns	0.39	ns	no	no	0.406		purified/relaxed
Dasyuromorphia	low	0.00	ns	0.00	ns	no	no	1.094		purified
	intermediate	0.97	ns	5.26	0.05	no	yes	0.131	0.255	purified
	high	0.00	ns	3.25	ns	no	no	0.250		purified
Diprotodontia	low	3.84	ns	5.05	0.05	no	yes	0.170		purified
	intermediate	3.72	ns	7.24	0.01	no	yes	0.153	0.141	purified
	high	4.32	0.05	2.51	0.05	yes	yes	0.000		very purified
Mammalia	low	1.15	ns	9.67	0.01	no	yes	0.474		purified
	intermediate	0.14	ns	19.66	0.00	no	yes	0.406	0.380	purified
	high	0.20	ns	18.62	0.00	no	yes	0.321		purified

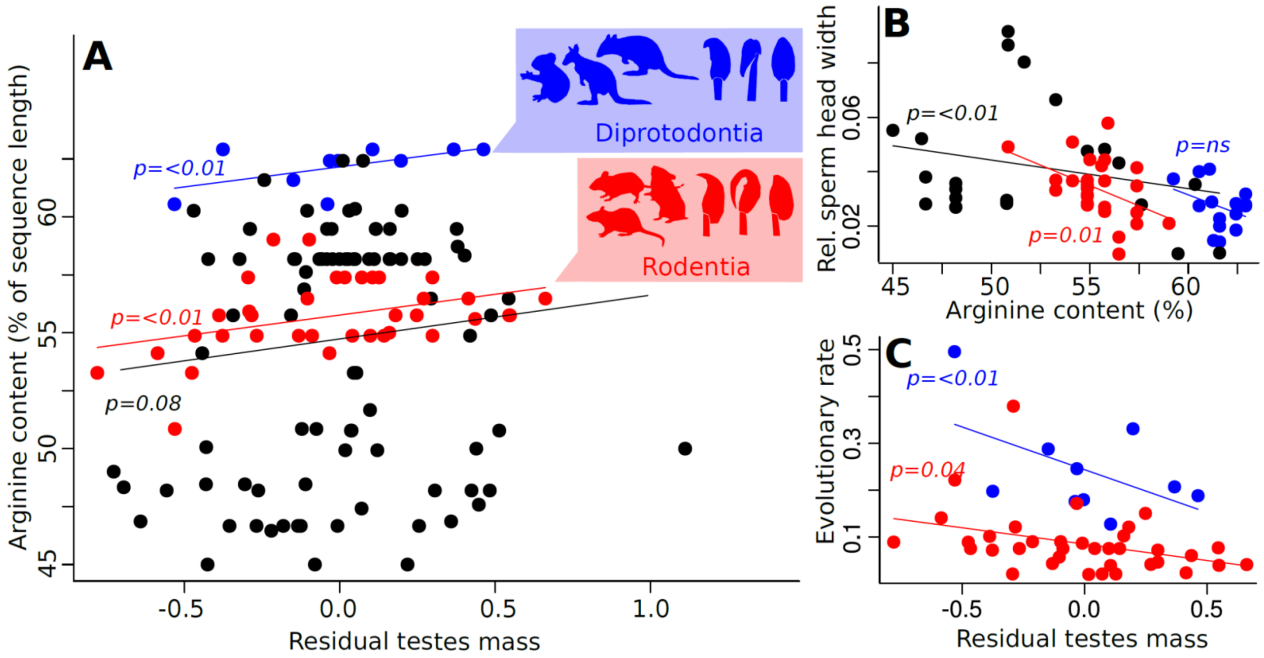

Figure 5.2. Visualization of PGLS regression analyses. (A) Regression for mammals between protamine 1 arginine content (as percentage of total sequence length) and corrected testes mass. (B) Regression for mammals between relative sperm head width and protamine 1 arginine content (as percentage of total sequence length). (C) Regression between protamine 1 evolutionary rate (root-to-tip ω) and corrected testes mass. Rodentia and Diprotodontia are identified in each plot. *P* values represent level of significance according to a PGLS regression analysis.

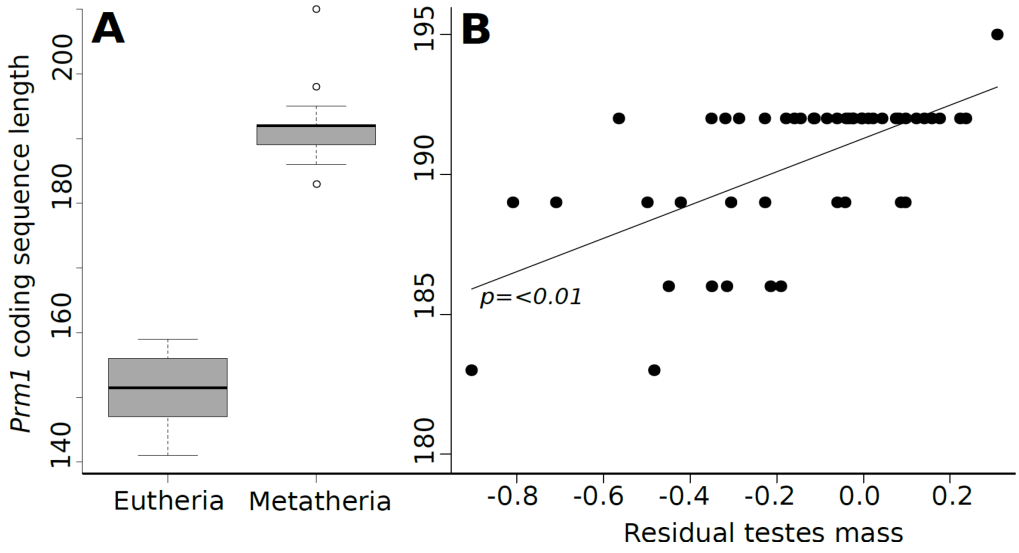

Figure 5.3. Sequence length in Metatheria. (A) Boxplot visualizing significant difference in protamine 1 coding sequence length between Eutheria and Metatheria. (B) Visualization of PGLS regression between metatherian protamine 1 coding sequence length and corrected testes mass. *P* value represents level of significance according to a PGLS regression analysis.

Associations with sperm head dimensions

In order to test for the effect of changes in the coding sequence of protamine 1 on sperm head dimensions, the evolutionary rate and the arginine content were used as independent variables in PGLS analyses, and relative head length (HL), relative head width (HW) and head elongation (HL/HW) were included as dependent variables. For Chiroptera, Artiodactyla and Dasyuromorpha data available for sperm head dimensions were insufficient for regression analyses. For Primates data available for relative HW were insufficient for regression analysis. We examined possible relationships across mammals and within clades.

Across mammals we found a significant negative correlation between arginine content and relative HW (Fig. 5.2B, ANNEX II Table S4). PGLS regessions showed a trend for positive correlation between root-to-tip ω and relative HL in Rodentia, and a significant positive correlation for Cetacea and Diprotodontia. In addition, a significant positive correlation was found in Rodentia and Diprotodontia between root-to-tip ω and HW. Finally, we found a significant negative correlation between arginine content in protamine 1 and relative HW in Rodentia and Cetacea and with relative HL in Cetacea (ANNEX II Table S4).

DISCUSSION

In this study we analyzed the evolution of protamine 1 in mammals and the possibility that postcopulatory sexual selection may be an important selective driver for this protein. We found evidence to suggest that an elevated evolutionary rate of the protamine 1 gene results in loss of arginine in most mammalian clades and, in turn, that higher arginine content associates with reduced sperm head width. There was also a clear relationship between postcopulatory sexual selection and arginine content in protamine 1. On the other hand, there was no relationship throughout mammals between postcopulatory sexual selection and the evolutionary rate of protamine 1, although this relationship was clearly observed in two clades, rodents and diprotodonts (which exhibited the highest arginine content in eutherians and metatherians, respectively). Within metatherians, the length of protamine 1 seems to be important as it was positively associated to sperm competition levels. Finally, we found that higher variability in inferred sperm competition levels seems to be related to a higher evolutionary rate and, in addition, a higher variability in arginine content (see Fig. 5.4 for a summary of the relationships found).

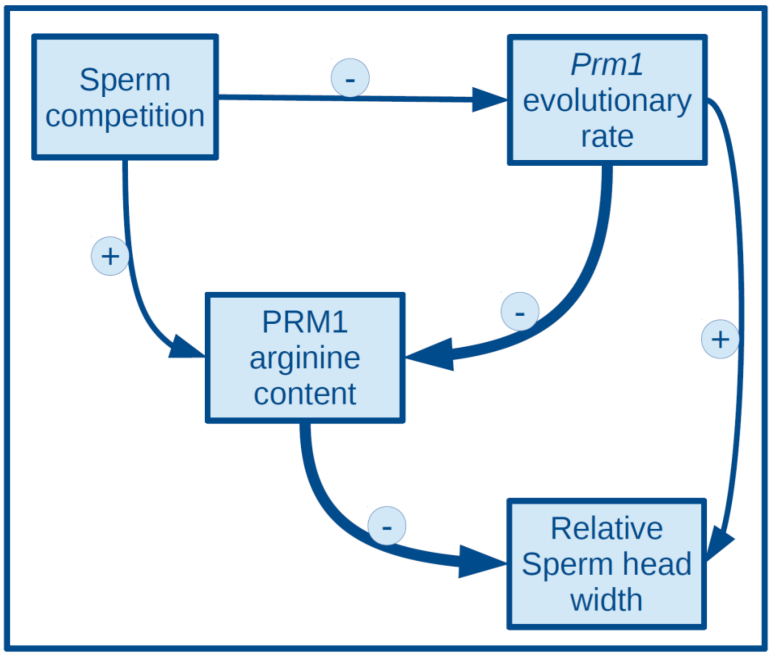

Figure 5.4. Schematic presentation of relationships found in this study. Arrows represent relationships found within mammalian groups. Bold arrows represent relationship found across all mammals.

Protamine sequence length is sexually selected in Metatheria but not in Eutheria

In this study we analyzed protamine 1 gene sequences of 237 mammalian species of eutherians and metatherians. Marsupial protamine 1 differs from the eutherian gene in several important ways and, in fact, is more similar in structure to bird protamines (Retief et al 1995a). Whereas eutherian protamine 1 contains 5 to 9 cysteine residues, which allow protamines to form complex tertiary structures by building disulfide bridges between and within protamines (Balhorn 1982, Oliva and Dixon 1991, Balhorn et al 1995), metatherian protamine sequences, like those of birds and fishes, lack these residues. An exception is the *Planigale* genus, which seems to have obtained up to 7 cysteine residues by convergent evolution (Retief et al 1995a). The lack of cysteine residues in most metatherians results in a less stable chromatin which is more easily condensed compared to that of eutherian mammals (Cummins 1980). Metatherian protamine 1 is significantly longer than eutherian's and contains more serine and tyrosine. N- and C-terminal serine residues, as well as the C-terminal threonine residues, are known phosphorylation targets in protamines (Balhorn 2007). It seems plausible that the higher serine content in metatherian protamine 1 might lead to a higher degree of phosphorylation. Due to these differences, metatherians are likely to bind chromatin in a slightly different manner than eutherians (Balhorn 1985). In fishes and birds the size of protamines was found to be an important factor in chromatin condensation (Oliva et al 1987, Oliva and Dixon 1991). Since metatherians, as birds and fishes, cannot rely on disulfide bridges to stabilize the chromatin, a longer protamine sequence might be necessary for efficient chromatin condensation. We therefore tested if sexual selection might be affecting protamine 1 sequence length within metatheria and found significant positive correlations between sequence length and inferred levels of sperm competition across metatherians and also within the two clades analyzed. Thus, sequence length seems to be a key factor in stabilizing chromatin in metatherian mammals as shown by its selection towards an increase of sequence length in species with higher levels of sperm competition.

Sperm competition maintains high arginine content of protamine 1 through sequence conservation

Protamine 1 is crucial for correct sperm chromatin condensation. Alterations in protamine content are linked to morphological abnormalities of the sperm head, increases in DNA damage, and decreases in sperm motility (Belokopytova et al 1993, Cho et al 2001, Aoki et

al 2005), and strongly affect male fertility (Cho et al 2003, Aoki et al 2005, Oliva 2006, Carrel et al 2007).

It was known that protamines with higher arginine content form more stable chromatin complexes, replace histones more efficiently and are more efficient in chromatin decondensation following fertilization (Ohtsuki et al 1996). We found arginine content of protamine 1 to be correlated positively with a proxy of sperm competition levels in mammals. This correlation was significant when Metatheria was not included in the analysis; methaterians have a significantly higher arginine content than eutherian mammals and might therefore affect the regression slope. However, when metatherians were included a clear trend was still observed. Species experiencing higher selective pressure through sperm competition showed higher arginine content in the protamine 1 amino acid sequence. Additionally, we found that an elevated evolutionary rate generally results in loss of arginine in mammals. The effect of sexual selection should therefore not only be detected on arginine content but also on the evolutionary rate. However this effect was only found in rodents and diprotodonts. The comparatively higher arginine content in these two clades could be the result of adaptation to sperm competition. But, if high arginine content is beneficial for species with high levels of sperm competition, and high evolutionary rate shows a general trend towards loss of arginine throughout mammals, why do we find the same trend only in rodents and diprotodonts when clades are examined?

The gene sequence of protamine 1 seems to be under purifying selective constraint across mammals and within all clades, with the exception of primates and cetaceans, which show evidence of relaxation. We analyzed the evolutionary rate not only on whole sequences but also performed an additional study calculating the possible positive selection at site level. We found signals for positive selection on site level in Chiroptera, Cetacea and in both metatherian clades. Previous studies show that, in rodents, changes in the protamine gene sequences and protamine expression ratio affect head size and shape (Lüke et al 2014a,b). Within the coding sequence of protamine 1 in mammals highly variable, as well as highly conserved regions can be found (Oliva and Dixon 1991, Rooney et al 2000, Wyckoff et al 2000). Arginine content seems to be conserved while its position seems to vary to a considerable extent (Rooney et al 2000). In primates the coding sequence was shown to be positively selected (Wyckoff et al 2000), while within rodents it has been shown to be functionally conserved, with directed positive selection on a few C-terminal sites (Lüke et al 2011). It seems that protamine 1 shows an unusual form of selection

which seems to be driven by adaptations to sexual selection (Oliva and Dixon 1991, Wyckoff et al 2000). Due to the importance in sperm form and function, as well as the protection of sperm DNA, protamine 1 might be affected by different types of selection resulting in a careful balance between conservation of function and adaptations to high sperm competition levels.

The main reason for sexual selection acting on protamine 1 is proposed to be its effect on sperm head shape. As an adaptation to high levels of sperm competition protamines might affect the head shape to become more hydrodynamically efficient and thus favoring higher sperm velocity. However, which type of sperm head is the most beneficial to ensure high sperm velocity is presently umknown. Considering the high variety of sperm head shapes and sperm sizes in mammals (Cummins and Woodall 1985, Roldan et al 1992, Pitnick et al 2009) it is plausible that a complex interplay between sperm metabolism, pattern of flagellar movement, total sperm size, hook shape and nucleus shape determine the ability of the sperm to swim faster (Malo et al 2006, Gómez-Montoto et al 2011, Tourmente et al 2013). For example, while we found that relative reduction in protamine 2 content seems to lead to a more beneficial head shape in mice in terms of reproductive fitness, we might not be able to extrapolate this relationship to other groups of species in which, for example, sperm heads do not have a hook or the flagellum shows a highly different beating pattern. Broader sperm heads might be beneficial for certain sperm morphologies, especially taking into account sperm head thickness. Sexual selection might therefore be affecting the arginine content and evolutionary rate of protamine 1 to varying degrees in different groups of species.

In general terms high evolutionary rate seems to lead to a loss of arginine. We found this relationship within all clades to varying degrees, except for cetaceans, which show a relatively lower arginine content than other eutherian clades. While higher levels of arginine coincide with both reduced sperm head width and length, sexual selection on arginine content could not be found in cetaceans. Further studies are needed to understand the role of sexual selection acting within this clade. Bats show a high level of arginine when compared to other eutherian mammals and we would expect this to be a result of sexual selection acting to maintain, or promote, a high arginine content. However, not enough data are available on body mass and testes mass in the literature to test for sexual selection in this clade. Thus, further studies are needed to understand the role of protamine 1 in bats.

Less selective constraints in clades with highly variable sperm competition levels?

When comparing selective constraints on protamine coding sequences between clades we observed conserved constraints in all groups except for primates and cetaceans, which showed a comparatively relaxed constraint. This seems to coincide with a comparatively higher variability in inferred levels of sperm competition in both groups. The clades' evolutionary rates correlated positively with clades' variability of sperm competition. While the clades' variability in arginine content seemed to increase with clades' variability of sperm competition, the clade mean arginine content decreased. Similarly, when looking at eutherians and metatherians in general, the lower selective constraints in metatherians seemed to coincide with lower variability in levels of sperm competition compared to eutherians. A group of species exhibiting big differences in sperm competition therefore also show bigger differences in their coding sequences resulting in the detection of a higher evolutionary rate and less selective constraints in the group. As a result, clades with high variability in sperm competition show higher variability and lower mean arginine content. Therefore, while the effect of sexual selection on evolutionary rate or arginine content seems to be difficult to detect by analyzing the relationship directly through regression analysis, the simple existence of an effect of sexual selection on protamine 1 evolutionary rate might be detected by analyzing levels of variation. This might lead to the conclusion that protamine 1 is sexually selected in primates and cetaceans while the nature and direction of the selection is complex and could be different between primate and ceatacean families.

Conclusions

We found evidence for sexual selection, in the form of sperm competition, maintaining a high arginine content across mammals, while changes in the protamine 1 coding sequence seem to result in loss of arginine. A higher arginine content seems to be associated with a reduction in sperm head width and was previously found to result in more efficient chromatin condensation. Due to a lack of cysteine residues, metatherian protamine 1 cannot rely on disulfide bridges to stabilize chromatin; this may be compensated by an increase in sequence length, which is sexually selected. Within mammalian clades sexual selection on evolutionary rate and arginine content was detected within Rodentia and Diprotodontia, while we found differences in selective pressures on sequences and codon sites between mammalian clades. Due to the wide variety of sperm head morphologies within mammals changes in arginine content and changes in protamine coding sequence

might be beneficial in some mammalian groups and disadvantageous in others. This would result in a complex evolutionary pattern of protamine 1 and difficulties in detecting sexual selection within mammalian groups. However, due to general sequence conservation, and the importance of arginine in chromatin condensation and head shaping, a general trend for sexual selection acting on arginine content, and its possible effect of sperm head width, has been identified in mammals. Since the interplay between evolutionary rate, arginine content, sperm head morphology, and swimming speed seems to be very complex, the effect of sexual selection on protamine 1 might not be readily detected in some groups. However, if an effect of sexual selection exists in general, it might be revealed when testing for an association between the level of variability in sperm competition and evolutionary rate.

ACKNOWLEDGEMENTS

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Lüke L, Tourmente M, Roldan ERS (0000) Sexual selection of protamine 1 in mammals. *Submitted for publication.*

CHAPTER 6

SELECTIVE CONSTRAINTS ON PROTAMINE 2 IN PRIMATES AND RODENTS

RESUMEN

Las protaminas son proteínas nucleares del espermatozoide con un papel crucial en la condensación de la cromatina. Su funcionamiento correcto está relacionado estrechamente con la morfología de la cabeza espermática y la fertilidad masculina. Se piensa que las protaminas se ven afectadas por un patrón complejo de restricciones selectivas. En estudios previos se demostró que la selección sexual afecta a las secuencias codificantes y a la expresión de las protaminas en roedores. En el presente estudio se analizaron las restricciones selectivas y la selección sexual que actúa en la secuencia del gen de protamina 2 en 53 especies de primates y roedores. Se focalizó en posibles diferencias en restricciones selectivas entre estos dos clados así como en los dos dominios funcionales de la protamina 2 (la protamina 2 madura y clivada). En forma adicional, se examinó si y cómo los cambios en la secuencia codificante de la protamina 2 puede afectar las dimensiones de la cabeza espermática. Se encontró que el dominio de la protamina 2 que se cliva y elimina durante el proceso de unión al ADN está bajo selección purificante en ambos clados, mientras que el dominio que permanece unido al ADN (protamina 2 madura) está seleccionado positivamente en primates y está bajo restricción relajada en roedores. Los cambios en la secuencia codificante de la protamina 2 clivada están asociados significativamente con el ancho y la elongación de la cabeza espermática en roedores. En forma contraria a lo anticipado, no se encontró un efecto marcado de la selección sexual en ninguno de los dos dominios o clados. Se concluye que la protamina 2 madura puede encontrarse libre para evolucionar bajo menor restricción debido a la existencia de la protamina 1 como una copia conservada y funcionalmente redundante. El dominio de protamina 2 clivada parece tener un papel importante en la forma de la cabeza espermática, aunque parece difícil dilucidar el efecto de la selección sexual hasta que se identifique qué fenotipo de cabeza espermática

(forma y tamaño) confiere ventajas para la función espermática en diferentes clados de mamíferos.

SUMMARY

Protamines are sperm nuclear proteins with a crucial role in chromatin condensation. Their correct functioning is strongly linked to sperm head morphology and male fertility. Protamines are thought to be affected by a complex pattern of selective constraints. Previous studies showed that sexual selection affects protamine coding sequence and expression in rodents. Here we analyze selective constraints and postcopulatory sexual selection acting on protamine 2 gene sequence in 53 species of primates and rodents. We focused on possible differences in selective constraints between the two clades as well as the two functional domains of protamine 2 (cleaved- and mature-protamine 2). Additionally, we assesed if and how changes in protamine 2 coding sequence may affect sperm head dimensions. The domain of protamine 2 that is cleaved off during binding to DNA was found to be under purifying selection in both clades, whereas the domain that remains bound to DNA (mature protamine 2) was found to be positively selected in primates and under relaxed constraint in rodents. Changes in cleaved-protamine 2 coding sequence are significantly associated with sperm head width and elongation across rodents. Contrary to expectations, a significant effect of sexual selection could not be found on either domain or clade. We conclude that mature-protamine 2 may be free to evolve under less constraint due to the existence of protamine 1 as a more conserved and functionally redundant copy. The cleaved-protamine 2 domain seems to play an important role in sperm head shaping, although sexual selection on its sequence may be difficult to detect until it is identified which sperm head phenotype (shape and size) confers advantages for sperm performance in different mammalian clades.

INTRODUCTION

The evolution, and underlying selective forces, of the high diversity observed in reproductive phenotypes are now the subject of considerable interest in evolutionary biology. Sperm competition, one of these selective forces, has been shown to affect the sperm phenotype of males competing for the fertilization of ova (Parker 1970) by driving adaptive changes of sperm morphology and function (reviewed in Birkhead and Moller 1998, Simmons 2001, Birkhead et al 2009). However, how these adaptations are produced through changes at the molecular level is not yet clearly understood.

Responses to high levels of sperm competition include increases in sperm numbers, which are achieved by an increase in testes mass relative to body size (Birkhead and Moller 1998, Birkhead et al 2009). Relative testes mass is strongly associated to levels of sperm competition (Birkhead and Moller 1998, Gomendio et al 1998, Birkhead et al 2009) and genetic paternity (Soulsbury and Dornhaus 2010). Thus, relative testes mass is widely used as proxy for level of sperm competition. Additional responses to high levels of sperm competition are increases in sperm swimming velocity, total sperm size and sperm quality (Gomendio and Roldan 1991, 2008, Gomez Montoto et al 2011, Tourmente et al 2011). The morphology of sperm cells, particularly the sperm head, are highy diverse among species (Cummins and Woodall 1985, Roldan et al 1992, Pitnick et al 2009). Changes in sperm head dimensions, size of the apical hook, and head shape are also known responses to high levels of sperm competition (Gomez Montoto et al 2011, Varea-Sánchez, 2014).

Studies on coding sequences of sperm proteins have identified proteins affected by selective pressure due to sperm competition. The evolutionary rate of coding sequences of two seminal fluid proteins (SEMG2 and SVS), two sperm surface proteins (ADAM 2 and ADAM18), and proteins of the acrosome (Zonadhesin and SPAM1) seem to be positively related to level of sperm competition in primates (Dorus et al 2004, Herlyn and Zischler 2007, Ramm et al 2008, Finn and Civetta 2010, Prothmann et al 2012). Other studies found a negative effect of sperm competition on the evolutionary rate of seminal fluid proteins in butterflies, or sperm nuclear proteins protamine 1 and protamine 2 in rodents (Walters and Harrison 2011, Lüke et al 2011).

Protamines are small, arginine-rich sperm nuclear proteins. They are crucial for sperm chromatin condensation through successive replacements of histones to transition nuclear proteins to protamines (Oliva and Dixon 1991). Protamine 1 is found throughout mammals,

whereas protamine 2 is found almost exclusively in primates and rodents. Evidence for the existence of protamine 2 gene, transcripts and, in some cases, mature protein is available in a few other mammalian species (Oliva and Dixon 1991, Oliva 2006, Balhorn 2007). Protamine 2, unlike protamine 1, codes for a precursor, which is processed by successive proteolytic cleavages at the time of sperm differentiation (Oliva and Dixon 1991, Oliva 2006). Protamine 2 processing occurs when DNA condensation is taking place and protamines are already bound to DNA (Lee et al 1995). A mature form of protamine 2 (hereafter, mature-Prm2) can be identified after cleavage. The role of the protamine 2 domain that is cleaved off (hereafter, cleaved-Prm2) from the precursor is not clear. The cleaved-Prm2 and mature-Prm2 are structually and functionally different (Lüke et al 2011, 2014a). Protamine 2 is believed to have evolved as the result of protamine 1 gene duplication with the latter resembling the sequence of the mature domain of protamine 2 (Krawetz and Dixon 1988, Lüke et al 2011). Both protamines 1 and 2 have DNA anchoring domains containing 3-7 arginine residues separated by uncharged amino acids (Balhorn et al 1999). The arginine residues neutralize the charge of the DNA backbone and may also play a role in the activation of egg casein kinase II after fertilization (Ohtsuki et al 1996).

Because of their important role during sperm chromatin condensation, alterations in protamine expression affect male fertility (Cho et al 2003, Aoki et al 2005, Oliva 2006, Carrel et al 2007). In men, changes in sperm protamine content reduce sperm number and sperm motility and affects sperm head morphology (Aoki et al 2005). Aberrant sperm chromatin condensation leads to larger heads and head abnormalities in men (Belokopytova et al 1993). In mice, an unbalanced protamine content associates with sperm DNA damage, sperm morphological abnormalities, and decreases in sperm motility (Cho et al 2001). Changes in protamine gene sequences and protamine expression ratios are linked to differences in head size and shape in muroid rodents (Lüke et al 2014a,b).

Protamines are thought to evolve fast, showing high structural heterogenity (Oliva and Dixon 1991, Wyckoff et al 2000). However, selective constraints are highly variable within the gene sequence and between taxa. Evidence of positive selection on the protamine 1 gene sequence has been detected in primates (Rooney et al 1999, Wyckoff et al 2000). Different selective constraints for protamine 1 and protamine 2 have been found in other mammalian species (Martin-Coello et al 2009, Lüke et al 2011). Within mammals protamines are thought to be diverse, especially in the C-terminal region, but they contain conserved regions that are also found in birds (N-terminal ARYR, SRSRSR phosphorylation site, 3 arginine clusters) (Queralt et al 1993). The high arginine content

was previously reported to be essentially conserved within the sequence, while the position of arginine residues seems to be highly variable (Rooney et al 2000). In a group of cricetid rodents, protamine 1 was shown to be under conserved selective constraint, with signs of positive selection restricted to specific codon sites. On the other hand, the two protamine 2 domains were shown to be under relaxed constraint on the way to degradation (Lüke et al 2011). Sperm competition was shown to reduce the relaxation acting on the gene sequence of protamine 2, resulting in a more conserved state of the gene in species with high levels of sperm competition (Lüke et al 2011).

In this study we examined the selective pressures potentially acting on protamine 2. Since protamine 2 is mainly expressed in rodents and primates this study concentrated on these clades. In addition, because the protamine 2 precursor actually contains two structurally and functionally different domains (cleaved-Prm2 and mature-Prm2) we analyzed them separately to examine the possibility that they may be under different selective pressures. Further to a comparison of selective pressures, we examined the possible effects of postcopulatory sexual selection (sperm competition) on the coding sequence and arginine content of mature protamine 2. We predicted that differences could exist in selective constraints on protamine 2 between primates and rodents. Since sexual selection was already shown to affect protamine 2 in cricetid rodents we anticipated signs of sexual selection for all rodents. Finally, we predicted that cleaved-Prm2 and mature-Prm2 could evolve under different selective regimes.

MATERIALS AND METHODS

Sequence data and phylogenetic tree

Protamine 2 gene sequences of primate and rodent species, as well as those available for other mammalian species, were taken from NCBI Genbank and previous publications (Lüke et al 2011) (ANNEX III Table S1), were visualized with Geneious 5.5.9 (Biomatters, http://www.geneious.com/) and trimmed to coding sequence based on NCBI Genbank information. Sequences were manually checked to ensure correct trimming. Translation alignments based on the muscle alignment algorithm implemented in Geneious 5.5.9 were performed and checked manually. Arginine frequencies were calculated using Geneious 5.5.9 (ANNEX III Table S1). The phylogenetic tree of the 53 mammalian species included in this study was constructed as a consensus of phylogenies available in the literature (ANNEX III Fig. S1 and references therein).

Phenotype data

Data on body mass, testes mass and sperm dimensions were obtained from the literature (ANNEX III Table S1 and references therein). Testes and body mass data were available for 46 of the 53 species for which sequence data were available. Data on sperm head width were available for 30 species and sperm head length for 44 species. Residual testes mass data were obtained from a regression analysis including body mass as independent variable and testes mass as dependent variable. Residual testes mass was only used for graphical representation of multiple regression results. Because total sperm length varies greatly among these species, and drag resulting from head size should be analyzed taking into account the length of the flagellum (Humphries et al 2008, Lüke et al 2014a), sperm head length and head width were each used as proportion of total sperm length (TSL) (hereafter, relative HL and relative HW).

Analysis of selective pressures

The nonsynonymous/synonymous substitutions rate ratio ($\omega = dN/dS$) is an indicator of selective pressure at the protein level, with $\omega = 1$ indicating neutral evolution, $\omega < 1$ purifying selection, and $\omega > 1$ diversifying positive selection (Goldman and Yang, 1994). To estimate rates of sequence evolution we used the application Codeml implemented in PAML 4 (Yang and Rannala, 1997, Yang, 2007). Likelihood-ratio-tests (LRT) were performed to test if the alternative model presents a better fit to the dataset against the null

model. For the Codeml codon frequency setting, as well as the setting for number of categories, we used the setting with the best fit for each analysis according to the preliminary likelihood-ratio-analysis. Branch lengths calculated in the model M0 “one-ratio” (see below) where used as input for subsequent models.

(a) Evolutionary rate (root-to-tip ω)

We used the free ratio model in codeml (PAML4) in order to obtain species-specific ω values. The free ratio model calculates ω freely for each branch in the tree. Species root-to-tip ω was subsequently calculated by addition of dN values and dS values from the root of the clade to the terminal species branch of the respective clade and taking the ratio of the sum to obtain the root-to-tip ω value (Lüke et al 2011, Montgomery et al 2011).

(b) Branch analysis

In order to obtain the evolutionary rate of clades and groups of species we computed the clade model comparing marked foreground branches against the unmarked background in the phylogenetic tree. Three models were computed: M0 ‘‘one ratio’’ in which all branches were constrained to evolve at the same rate; MCfixed ‘‘two-ratio, foreground fixed’’ where the background clade ω was allowed to be estimated freely while the foreground ω was restrained to a value of ω = 1; and MC ‘‘two ratio’’ model which estimates for both background and the Cricetidae clade a free and independent ω. To test if the foreground evolves at a significantly different rate than the background we compared M0 versus MC by means of LRT. If foreground ω was significantly higher than 1 (LRT significant for MCfixed vs MC and ω > 1) we assumed positive selection acting on the foreground branches at whole sequence level. If foreground ω was significantly lower than 1 (LRT significant for MCfixed vs MC and ω > 1) we report purifying selection acting on the branch at whole sequence level. Relaxed selective constraint for the foreground branch is assumed if foreground evolves at a significantly different ω than the background (M0 vs MC), and this ω was not significantly different from 1 (MCfixed vs MC) (Yang, 1998).

(c) Branch-site analysis

To test evolution among coding sequences and infer amino acids under positive selection for marked foreground branches in contrast to the unmarked background two models were computed. BSfixed “branch-site model A, foreground fixed” in which the codon site ω for background branches is allowed to be computed freely and BS “branch-site model A” in which codon sites in both foreground and background were computed freely (Zhang et al 2005). Evidence of the existence of positively selected codon sites (PSS) is reported if LRT between BSfixed and BS is significant and sites significantly belonging to the positive

selected site category are reported by the model.

Phyologenetically corrected regression analysis (PGLS)

To test for correlations between variables we employed the phylogenetic generalized least squares approach (PGLS) (Freckleton, 2002). Body mass and testes mass were included as independend variables in a multiple PGLS regression as a proxy for sperm competition (hereafter: relative testes mass). Analyses of associations between genetic and morphometric traits also took into account that such traits are not independent from their phylogenetic history (Harvey and Pagel, 1991). The PGLS approach has been shown to be a powerful tool to detect associations of this kind (Rholf 2001), and it has been used in earlier studies in combination with the root-to tip dN/dS method showing genetic-morphometric associations (Montgomery et al 2011, 2012, Pointer et al 2012, Lüke et al 2014a). We performed PGLS analysis using the R-package Caper.

RESULTS

Sequence properties

Sequence similarity and arginine content were compared between primates and rodents (ANNEX III Table S1). Cleaved-Prm2 sequence was significantly longer in primates ($t_{24.08}$ = 7.22, $P < 0.001$) whereas mature-Prm2 was significantly longer in rodents ($t_{20.5}$ = -13.5, $P < 0.001$). No significant difference was found in mature-Prm2 arginine content between primates and rodents ($t_{22.87}$ = -0.13, $P = 0.9$).

Selective pressures across species

We tested for the general mode of selection acting on protamine 2 domains in all species. Mammalian species other than rodents and primates were included to provide a background for comparison between primates and rodents. To obtain the backround pressure acting on the whole sequence across all species we calculated the evolutionary rate (ω) for the whole tree on the entire sequence (Codeml PAML4 model M0 as explained in Materials and methods). The evolutionary rate calculated across all species in model M0 for cleaved-Prm2 was ω = 0.54, and for mature-Prm2 it was ω = 1.18.

Comparison of selective pressures

To compare selective pressures for the whole sequence and for directed selective pressure on codon sites we employed a branch analysis and a branch-site analysis, first marking primates as foreground against the other species as background, and then marking rodents as foreground against the other species as background.

The branch analysis of cleaved-Prm2 comparing primates and rodents showed no differences between clades. Selective pressures did not differ significantly from the background for the two clades (primates and rodents: M0 vs MC not significant, M0 ω = 0.54). For mature-Prm2, primates showed significantly lower selective constraints than rodents (primates: M0 vs MC significant, MC ω = 3.12) whereas rodents did not evolve with a significantly different rate than the background (Rodents: M0 vs MC not significant, M0 ω = 1.18) (Table 6.1).

For cleaved-Prm2, the calculated evolutionary rate was significantly different from 1 for both clades (MCfix vs MC significant, M0 ω = 0.54) which, in combination with the low calculated evolutionary rate, suggests the domain to be under purifying selection in both primates and rodents. Results of branch analysis for mature-Prm2 in primates suggest positive selection. According to LRT, the calculated ω was significantly greater than 1 (MCfix vs MC significant, MC ω = 3.12). In rodents the calculated ω of mature-Prm2 did not differ significantly from 1 (MCfix vs MC significant, M0 ω = 1.18) suggesting relaxed constraint for this domain in rodents (Table 6.1).

The branch-site test showed no directed selection on codon sites for cleaved-Prm2 in primates (BSfixed vs BS non significant), while for rodents one codon site of cleaved-Prm2 was shown to be positively selected (BSfixed vs BS significant)(Table 6.1). For mature-Prm2, both primates and rodents showed significantly positively selected codon sites within the alignment (BSfixed vs BS significant)(Table 6.1).

The root-to-tip ω calculated for all species is shown in Table S1 (ANNEX III).

Table 6.1 Summary of results for branch analysis and branch-site analysis of protamine 2 domains of primates and rodents.

Sequence	**cleaved Prm2**		**mature Prm2**	
Clade (foreground)	Primates	Rodentia	Primates	Rodentia
LRTs for selection over whole sequence:				
2d(M0-C)	0.02	1.94	25.86	1.49
p	ns	ns	0.00	ns
2d(Cfix-C)	8.74	15.42	25.60	0.01
p	0.01	0.00	0.00	ns
LRTs for directed selection on sites:				
2d(M1-BS)	1.67	5.27	62.21	26.10
p	ns	ns	0.00	0.00
2d(BSfix-BS)	0.00	5.27	96.75	60.63
p	ns	0.05	0.00	0.00
Clade ω as calculated by branch analysis model:				
ω	0.530	0.420	3.120	0.980
Proportion of sites in ω site classes:				
0	0.23	0.23	0.18	0.21
1	0.64	0.74	0.30	0.75
2a	0.04	0.01	0.20	0.01
2b	0.10	0.02	0.32	0.03
Positively selected sites (BEB p<0.05):				
PSS	-	26G	1Q, 4C, 5Y, 6G, 7Y, 11L, 24Q, 25R, 29R, 44R, 45N, 51R, 55T, 61T	64-,72-
Interpretation:				
Selection over whole seqence	conserved	conserved	positive	relaxed
Directed selection on sites	no signal	positive	positive	positive
Sexual selection	not detected	not detected	not detected	not detected

LRT= Likelihood ratio test, ω = clade omega as caculated by branch analysis, if LRT of M0 versus MC significant MC omega is reported if LRT non significant M0 omega is reported. PSS=positively selected sites. Ω site classes: 0: 0<ω<1 for foreground and background branches, 1: ω=1 for foreground and background branches, 2a: 0<ω<1 for background and ω>1 for foreground, 2b: ω=1 for background and ω>1 for foreground.

Sexual selection

To test for sexual selection on protamine 2 coding sequences in primates and rodents we chose the phylogenetic generalized least squares (PGLS) regression analysis as described below. The root-to-tip ω as well as and arginine content (as percent of sequence length) were included as dependent variables against body mass and testes mass as independent variables (relative testes mass, as proxy for sperm competition). We tested for an effect of evolutionary rate on arginine content by including it as dependent variable and root-to-tip ω as independent variable in the PGLS regression.

No significant correlations with residual testes mass for either protamine domains root-to-tip ω or mature-Prm2 arginine content was found in primates or rodents. Arginine content was not correlated with root-to-tip ω (ANNEX III Table S2).

Relationships with sperm head dimensions

We tested for the effect of changes in the coding sequence of protamine 2 domains on sperm head dimensions. The evolutionary rate was used as independent variable in PGLS analyses, with relative head length (HL), relative head width (HW), and head elongation (HL/HW) used as dependent variables. For primates, data available for relative HW were not sufficient for regression analysis. PGLS regressions showed no significant correlations with relative HL or head elongation in primates (ANNEX III Table S2). In rodents, PGLS regressions showed a significant positive correlation between cleaved-Prm2 root-to-tip ω and relative HW, and a significant negative correlation between cleaved-Prm2 root-to-tip ω with head elongation (Fig. 6.1, ANNEX III Table S2). No significant correlations were found with mature-Prm2.

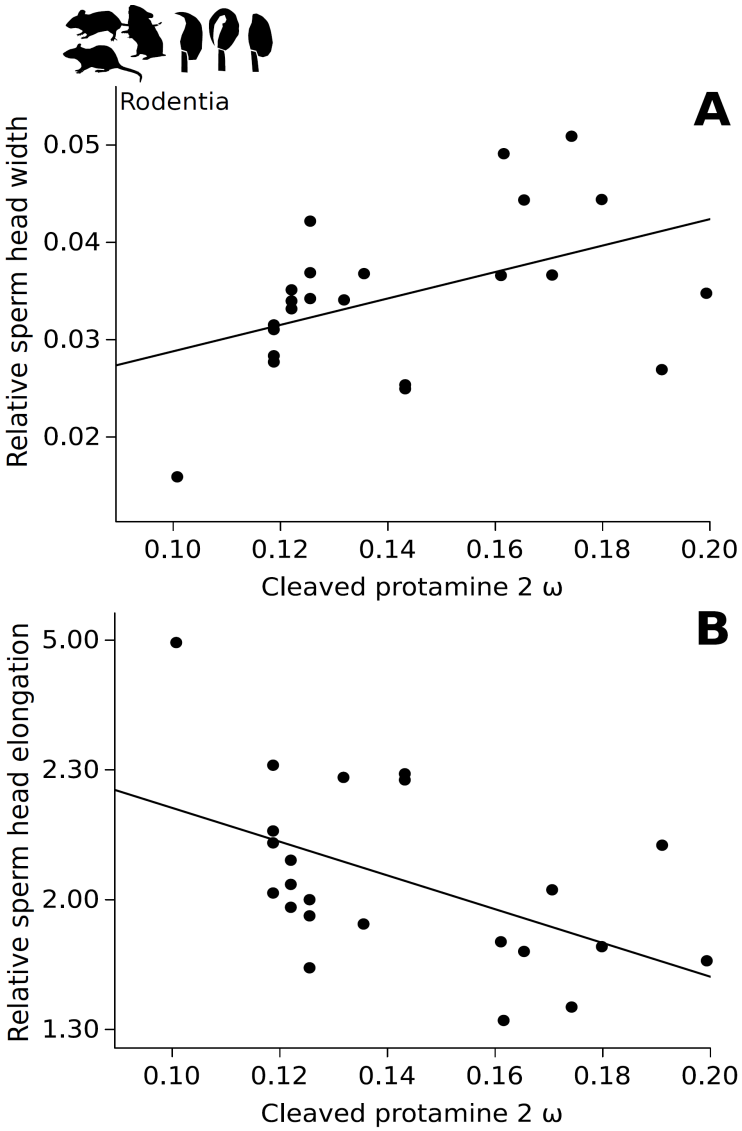

Fig. 6.1 Visualization of significant PGLS regression results for **(a)** Relationship in rodents between cleaved protamine 2 ω (root-to-tip ω) with sperm head width (relative to total sperm length) and **(b)** Relationship in rodents between cleaved protamine 2 ω (root-to-tip ω) with sperm head elongation (sperm head length divided by sperm head width).

DISCUSSION

In this comparative study, focusing on possible selective constraints acting on the protamine 2 gene, we were able to demonstrate significant differences between evolutionary rates of primate and rodent protamine 2 as well as between the cleaved and the mature protamine 2 domains. In primates and rodents cleaved protamine 2 is conserved although one site was found to be positively selected in rodents. Mature protamine 2 is under relaxed constraint in rodents and positively selected in primates. Additionally, we found directed positive selection on specific codon sites of mature protamine 2 in both primates and rodents. A previous study concentrating solely on the cricetid family of rodents (Lüke et al 2014a) showed how changes in the cleaved protamine 2 gene sequence associates with sperm head width and elongation. Here we present evidence for this relationship to be true across rodents. No signal of sexual selection was found for primates or rodents.

Differences in selective constraints between taxa

Studies in rodents demonstrated that protamine gene sequences and protamine expression ratios influence sperm head size and shape (Lüke et al 2014a,b). The gene sequence of protamine 1 seems to be highly variable in mammals although highly conserved regions can also be identified (Oliva and Dixon 1991, Rooney et al 2000, Wyckoff et al 2000). Protamine 1 seems to have an unusual form of selection which seems to be driven by sexual selection (Oliva and Dixon 1991, Wyckoff et al 2000). This complex pattern of selective constraints and sexual selection could be a consequence of the importance of protamine 1 for sperm form and function resulting in a delicate balance between conservation of function and adaptations to high sperm competition levels. We expected to find an even more complex pattern of evolution in protamine 2 due to the existence of two domains in this protein. We were able to show differences in selective constraints between primates and rodents, especially for mature protamine 2, which is positively selected in primates and is under relaxed constraint in rodents. Cleaved protamine 2 is conserved in both clades. This result is important in connection to the proposed functional redundancy of protamine 1 and mature protamine 2. Mature protamine 2 is thought to be the result of protamine 1 gene duplication (Krawetz and Dixon 1988, Lüke et al 2011) and despite the proposed slight differences in function, mainly associated to the process of DNA condensation (Lüke et al 2014a), the function of mature

protamine 2 is essentially redundant to that of protamine 1. This might be an explanation for the comparative lack of selective constraint of mature protamine 2. Due to the existence of two protamines, one may bee “free” to adaptively evolve, or be relaxed, while the other is more conserved with a more directed pattern of positive selection on specific codon sites to ensure proper function (Lüke et al 2011) (Table 6.2).

Table 6.2 Comparison of selective constraints in mature protamine 2 and protamine 1 of rodents and primates.

Clade	mature *Prm2*	*Prm1*
Primates	positive selection	relaxed constraint
Rodents	relaxed constraint	purifying selection

Results on *Prm1* are from unpublished work by L. Lüke, M. Tourmente and E.R.S. Roldan.

No sexual selection detected for rodents or primates

We did not find signs of sexual selection acting on protamine 2 domains of primates or rodents. In a previous study, postcopulatory sexual selection (sperm competition) was found to halt the degree of relaxation in protamine 2 of cricetid rodents. Here, analyzing murids and cricetids together, this relationship was not observed. Sexual selection acting on protamines is proposed to be mainly due to their effect on sperm head shape. As an adaptation to high levels of sperm competition protamines seem to affect the head shape to become more hydrodynamically efficient favouring sperm velocity. However, it is not well known how changes in sperm head shape influence sperm velocity. Given the considerable variations in sperm head shapes and sizes in mammals (Cummins and Woodall 1985, Roldan et al 1992, Pitnick et al 2009), as well as the diversity in flagellar beating patterns and environments present in the female tract, it is fair to assume that adaptation of sperm head shape is the result of a complex interplay between these factors (Malo et al 2006, Gómez-Montoto et al 2011, Tourmente et al 2013). Wider sperm heads, for example, might be advantageous for certain sperm morphologies. Sexual selection effects may therefore be variable and even contradictory between different groups of species. A study comparing groups of species at deeper taxonomic levels and including more species might shed more light on the role of sperm competition on protamine 2 evolution. As protamine 1, mature protamine 2 is very rich in arginine. The DNA anchoring domains contain 3-7 arginine residues separated by uncharged amino acids (Balhorn et al

1999). Arginine neutralizes the charge of the DNA backbone and may play a role after fertilization (Ohtsuki et al 1996). For protamine 1, sexual selection seems to be targeting especially the arginine content of the gene. Sperm competition seems to maintain high arginine content of protamine 1 through sequence conservation. Species experiencing higher selective pressure through sperm competition show higher arginine content in the protamine 1 amino acid sequence (L. Lüke, M. Tourmente and E.R.S. Roldan, unpublished results). Unlike what was observed for protamine 1, we did not find a relationship between sexual selection and arginine content in mature protamine 2. This might be explained by the fact that the arginine content of mature protamine 2 seems to be stable across primates and rodents, showing very low variability, leading to the conclusion that it is highly conserved.

Cleaved protamine 2

Our results show that cleaved protamine 2 is conserved in rodents and primates, although we found one codon site to be positively selected in rodent cleaved protamine 2. In agreement with previous studies, we found that changes in the cleaved protamine 2 coding sequence associate with wider and more elongated sperm heads in rodents (Lüke et al 2014a). The role of cleaved protamine 2 is not yet clear but its conservation and the apparent influence it has on sperm head shape speaks for an important function especially in relation to sperm competitiveness. The unprocessed protamine 2 precursor binds to DNA and, while bound, is cleaved over a period of several days until only mature protamine 2 is left bound to DNA (Carre-Eusebe et al 1991, Chauvière et al 1992). Sperm chromatin condensation was shown to coincide temporally with the start of protamine translation and posttranslational processing (Kierzenbaum and Tres 1975, Lee et al 1995). Therefore, it was proposed that the cleaved protamine 2 domain may have a more important role during the actual process of chromatin condensation than mature protamine 2 (Lüke et al 2014a). In order to understand its role in sperm competitiveness and male fertility the function of cleaved protamine 2 should be studied in more detail.

Conclusions

As predicted, we found significant differences in selective constraints of the two protamine 2 domains (cleaved- and mature-protamine 2) as well as differences between the two clades studied (primates and rodents). Mature protamine 2 is generally relaxed in rodents with directed positive selection on sites and positively selected in primates. Mature

protamine 2 exhibits less constraint than its functionally redundant partner protamine 1. We propose that mature protamine 2 is free to evolve adaptively, or under less constraint, due to the existence of a more conserved, functional copy with redundant functional properties.

On the other hand, cleaved protamine 2 is conserved in both clades with signs of directed positive selection in rodents. We were also able to demonstrate that changes in cleaved protamine 2 affect sperm head width and elongation across rodents. This domain seems to play an important role in the process of sperm chromatin condensation and sperm head shaping. Further studies should focus on the function of this important protamine 2 domain. The fact that sexual selection was not detected in rodents or primates might be the result of differential interactions or trade-offs between sperm traits and its environment. A comparative study including a broader range of species might explain the complex processes of sexual selection acting on protamine 2.

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CHAPTER 7

SEXUAL SELECTION ON PROTAMINE AND TRANSITION NUCLEAR PROTEIN EXPRESSION IN MOUSE SPECIES

RESUMEN

La selección sexual postcópula, en la forma de competición espermática, influye en la evolución de proteinas reproductivas masculinas en mamíferos. La relación entre la competición espermática y la evolución reguladora, sin embargo, permanece inexplorada. Las protaminas y las proteinas nucleares de transición están involucradas en la condensación de la cromatina espermática y se estima que afectan la forma de la cabeza espermática. Una cabeza espermática hidrodinámicamente eficiente permite una velocidad de natación rápida y, por tanto, unos espermatozoides más competitivos. Los estudios comparativos previos en roedores han documentado una asociación significativa entre el nivel de competición espermática (estimado mediante la masa testicular relativa) y la evolución de secuencias de ADN tanto en las secuencias codificantes como en las del promotor de protamina 2. En este estudio, se investiga la influencia de la selección sexual sobre la expresión de mRNA de protaminas y proteínas nucleares de transición. Se examinó también la relación entre la expresión génica relativa y la forma de las cabezas espermáticas analizadas mediante morfometría geométrica. Se encontró que las especies con niveles más altos de competición espermática expresan menos protamina 2 en relación a protamina 2 y proteínas nucleares de transición. Además, se encontró una asociación significativa entre la expresión relativa de protamina 2 y la forma de la cabeza espermática. La reducción de la abundancia relativa de protamina 2 podría incrementar la capacidad competitiva de los espermatozoides de ratón, probablemente afectando la forma de la cabeza espermática. Los cambios en las secuencias reguladoras de los genes parece por tanto constituir la base de las respuestas evolutivas a la selección sexual en estas proteínas.

SUMMARY

Postcopulatory sexual selection in the form of sperm competition is known to influence the evolution of male reproductive proteins in mammals. The relationship between sperm competition and regulatory evolution, however, remains to be explored. Protamines and transition nuclear proteins are involved in the condensation of sperm chromatin and are expected to affect the shape of the sperm head. A hydrodynamically-efficient head allows for fast swimming velocity and, therefore, more competitive sperm. Previous comparative studies in rodents have documented a significant association between the level of sperm competition (as measured by relative testes mass) and DNA sequence evolution in both the coding and promoter sequences of protamine 2. Here, we investigate the influence of sexual selection on protamine and transition nuclear protein mRNA expression in the testes of eight mouse species that differ widely in levels of sperm competition. We also examined the relationship between relative gene expression levels and sperm head shape, assessed using geometric morphometrics. We found that species with higher levels of sperm competition express less protamine 2 in relation to protamine 1 and transition nuclear proteins. Moreover, there was a significant association between relative protamine 2 expression and sperm head shape. Reduction in the relative abundance of protamine 2 may increase the competitive ability of sperm in mice, possibly by affecting sperm head shape. Changes in gene regulatory sequences thus seem to be the basis of the evolutionary response to sexual selection in these proteins.

INTRODUCTION

When females mate promiscuously, the sperm of rival males compete for the fertilization of available ova (Parker 1970). Postcopulatory sexual selection mediated by sperm competition has a profound influence on male reproductive traits across a wide range of taxa (reviewed in Birkhead and Møller 1998, Simmons 2001, Birkhead et al 2009). In mammals, key traits affected by sperm competition include sperm quality parameters (Gómez Montoto et al 2011a), processes that prepare sperm to interact with the oocyte (Gomendio et al 2006), sperm design (e.g., overall size, head shape and dimensions (Roldan et al 1992, Breed and Taylor 2000, Immler et al 2007, Tourmente et al 2011a), and sperm swimming velocity (Gomendio and Roldan 1991, Gomendio and Roldan 2008, Toumente et al 2011a). Several lines of evidence suggest that sperm head shape is particularly important in competitive situations. For example, the size and curvature of the apical hook of rodent sperm heads is thought to be associated with levels of sperm competition (Immler et al 2007, but see Firman and Simmons 2009). Likewise, head shape may affect the hydrodynamic efficiency of spermatozoa. Head elongation, which may reduce drag, associates with faster sperm swimming velocity (Tourmente et al 2011a). Faster sperm are more likely to succeed in fertilization (Cummins and Yanagimichi 1982).

To date, most work on the molecular evolution of male reproductive genes has focused on protein-coding regions (Swanson and Vacquier 2002, Turner and Heokstra 2008). A number of studies have found a positive relationship between sequence divergence of these genes and levels of sperm competition, and several such genes show evidence of positive selection in coding regions (Wyckoff et al 2000, Kingan et al 2003, Dorus et al 2004, Ramm et al 2008, Finn and Civetta 2010, but see Lüke et al 2011, Walters and Harrison 2011). However, a positive correlation between sequence divergence in the promoter region of protamine *2* and relative levels of sperm competition in house mice and their close relatives (Martin-Coello et al 2009) suggests that regulatory changes may also contribute to species differences in sperm competitive ability. Surprisingly, despite order of magnitude differences in the absolute and relative expression levels of protamines and associated transition nuclear proteins across eutherian mammals (Kleene and Bagarova 2008), the relationship between sperm competition and gene expression remains largely unexplored.

Protamines and transition nuclear proteins are integral to chromatin remodelling and condensation during the final stages of spermatogenesis. This nuclear reshaping in

postmeiotic spermatids affects the overall shape of the sperm head which, in turn, may influence hydrodynamic efficiency, resulting in an increase in sperm swimming speed and more competitive sperm. Notably, sperm from transition nuclear protein-deficient mice perform poorly in some competitive assays (Nayernia et al 2003). Whereas protamines (PRM1 and PRM2 in most eutherian mammals) bind directly to DNA in the nucleus of elongating spermatids and mature spermatozoa (Brewer 1999), transition nuclear proteins (TNP1 and TNP2) are involved in intermediate stages in the replacement of histones by protamines (Yu et al 2000, Meistrich et al 2003). Protamines remain associated with sperm chromatin in the oocyte and influence the rate of nuclear decondensation, a trait associated with embryonic survival (Perreault et al 1988, Aoki and Carrell 2003, Cho et al 2003, McLay and Clarke 2003).

Protamine and transition protein mRNAs are highly co-expressed in round spermatids (Mali et al 1989, Hecht 1993, Oliva 2006, Balhorn 2007), and the protein products of both gene families exhibit significant overlap in elongating spermatid nuclei (Mali et al 1989, Meistrich et al 2003). TNP1 and TNP2 seem to perform partially redundant functions: only double TNP1/TNP2 mouse knockouts are completely sterile (Meistrich et al 2003). However, deletion of either transition protein results in incomplete PRM2 processing and defective chromatin condensation (Zhao et al 2001, Yu et al 2000). This, together with the co-localization of mRNAs and mature proteins, strongly suggests that functional interactions between protamines and transition proteins are necessary for normal sperm development.

In mice and humans, both PRM1 and PRM2 are essential for male fertility (Cho et al 2001). Strikingly, although the relative abundance of PRM1 and PRM2 proteins differs widely across mammals (from 0 to 77% PRM2) (Corzett et al 2002), disruption of species-specific protamine ratios causes fertility defects comparable to gene knockouts (Cho et al 2001, Haueter et al 2010). In human males, for example, protamine imbalance can result in reduced sperm concentration and motility, and in abnormal head morphology, an indicator of deficits in chromatin condensation (de Yebra et al 1998, Carrell and Liu 2001, Aoki et al 2005). In particular, incomplete processing of the PRM2 precursor is associated with sperm dysfunction (de Yebra et al 1998, Torregrosa et al 2006), and PRM2-deficient sperm are characterized by incomplete nuclear condensation and increased DNA damage (Carrell et al 1999, Cho et al 2001, Torregrosa et al 2006), defects that can lead to embryonic mortality (Cho et al 2003). Thus, protamine ratios play a large role in sperm head morphology, a phenotype important for competitive ability both before and during

fertilization. This suggests that sexual selection mediated by sperm competition should act on protamine ratios, resulting in an association between species differences in levels of sperm competition and protamine expression.

Here, we investigate the influence of sexual selection on protamine and transition nuclear protein mRNA expression in the testes of eight closely related species in the genus *Mus*. These species exhibit a wide range of relative testes mass, a robust proxy for different levels of sperm competition (Birkhead and Møller 1998, Birkhead et al 2009), and differ in sperm traits associated with competitive ability (Roldan et al 1997, Martin-Coello et al 2009, Gómez Montoto et al 2011a,b). Moreover, evolution of the *Prm2* promoter in seven of the same species is consistent with stronger selection in taxa with higher inferred levels of sperm competition (Martin-Coello et al 2009). This provides specific motivation for studying the relationship between protamine expression and sperm competition in *Mus*. Given the functional relationship between protamines and transition proteins, and the role of transition proteins in PRM2 processing, we expected that transition nuclear protein expression should co-vary with species differences in protamine expression. Because protamines and transition nuclear proteins are involved in the condensation of sperm chromatin, and are expected to affect the shape of the sperm head, we also assessed the relationship between gene expression and sperm head shape.

MATERIALS AND METHODS

Species

This study included 8 species in the genus *Mus*: *M. caroli, M. castaneus, M. domesticus, M. macedonicus, M. musculus, M. pahari, M. spicilegus,* and *M. spretus* (4 to 5 males per species). This group of species shows diverse levels of sperm competition, as inferred from their differences in relative testes mass (Table 1). Large testes in relation to body mass (relative testes mass) is a strong predictor of high sperm competition levels in many taxa (reviewed in Birkhead and Møller 1998, Gomendio et al 1998, Birkhead et al 2009), and relative testes mass is correlated with genetic paternity (i.e., percentages of multiple paternity) in mammals in general (Soulsbury 2010), and rodents in particular (Ramm et al 2005). Therefore, relative testes mass is used in this study as a robust proxy for sperm competition levels.

Individuals were purchased from the Institut des Sciences de l'Evolution-Montpellier, CNRS-Universite de Montpellier II. Males were kept in our animal facilities in individual cages under standard laboratory conditions in environmentally-controlled rooms (20 - 24ºC) on a 14 h light - 10 h darkness photoperiod, and were provided with food and water ad libitum. All animal handling was done following Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65.

Testes collection and relative testes mass

Animals were sacrificed at an age of 2 to 4 months by cervical dislocation and were immediately weighed and dissected. Testes were removed, weighed, flash-frozen in liquid nitrogen, and stored at -80ºC. All dissection instruments and areas were cleaned with RNase AWAY® (Molecular BioProducts, Thermo Fisher Scientific, San Diego, CA) before use. Relative testes mass was calculated based on the rodent power function, following the method in Kenagy and Trombulak (1896).

RNA extraction and cDNA synthesis

RNA was extracted in a sterile vertical laminar flow hood using either the RNeasy Plus kit (Qiagen) or the E.Z.N.A® Total RNA kit I (Omega, Madrid, Spain) following the manufacturer's recommendations. All instruments and surface areas were cleaned with RNase AWAY®. RNA concentration and purity were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Madrid, Spain) and cDNA was synthesized the same day from 10 µg of RNA, using the Superscript III First Strand Synthesis Kit with oligo(dT) (Invitrogen, Barcelona, Spain) according to the manufacturer's recommendations. cDNA concentration and purity were determined using a NanoDrop1000 spectrophotometer and samples were stored at -20ºC.

Quantitative PCR (qPCR)

Expression levels for *M. musculus, M. spretus*, *M. spicilegus,* and *M. pahari* were determined at the University of Arizona in Tucson using a MyiQ2 light cycler (Bio-Rad), and expression levels for *M. domesticus, M. castaneus, M. macedonicus*, and *M. caroli* were determined at the Museo Nacional de Ciencias Naturales in Madrid using a CFX96 Real Time System / C1000 Thermal Cycler (Bio-Rad). To check the consistency of results obtained using different cyclers, assays for the standard gene (see below) were run by the same person (LL) with a set of testes samples taken from the same individuals used in

both Tucson and Madrid, using exactly the same protocol. Results were consistent across locations (e.g., *Mus musculus* individual 1 (Tucson, right testis): average C_T (± SD) = 12.94 (0.02); *Mus musculus* individual 1 (Madrid, left testis): average C_T (± SD) = 12.89 (0.07)).

Primers were designed in Primer3 (v. 0.4.0) to amplify a product between 70 and 150 bases across an exon-exon junction. Protamine primers were placed in sequences that are invariant across all species in this analysis. Transition protein primers were placed in sequences that are conserved between *Mus* and *Rattus*, and therefore are unlikely to vary among closely related *Mus* species. Primer sequences and amplicon sizes are provided in supplementary table S1 (ANNEX IV). Each qPCR run included one individual of each species with three technical replicates for the four experimental genes (*Prm1, Prm2, Tnp1,* and *Tnp2*), and two technical replicates for the standard gene (*18SrRNA*). qPCR reactions were run in 96-well plates with an end volume of 16 µl per sample containing 8 µl SYBR green Master Mix (Invitrogen), 15 ng of each primer and 50 ng/µl of cDNA. The conditions of the thermocycler program consisted of an initial denaturation of 95ºC for 10 min, 40 cycles of 95ºC for 15 sec and an annealing and elongation stage of 62ºC for 1 min. Melt curve analysis was performed at the end of each run to check for multiple peaks, indicative of non-specific amplification.

Analysis of expression data

Cycle threshold data (C_T) were normalized relative to *18SrRNA* for each plate (ΔC_T). To avoid statistical analysis using a dataset of mixed negative and positive values, data were transformed by adding a constant based on the lowest ΔC_T value. Expression ratios and percentages were calculated from transformed individual ΔC_T values (*M. domesticus n* = 4, all other species *n* = 5), and median values were obtained for each species. Because of the expectation that relative expression levels may be of greater functional significance than absolute expression levels (see above), we calculated ratios (*Prm1/Prm2, Tnp1/Tnp2, Prm/Tnp, Prm2/Tnp*) and proportions (*Prm2/Prm, Prm2/*(*Prm*+*Tnp*), *Prm1/* (*Prm*+*Tnp*)), where *Prm* refers to the combined expression of *Prm1* and *Prm2*, and *Tnp* refers to the combined expression of *Tnp1* and *Tnp2*. To obtain a measure of variability between individuals and species, as well as for individual genes, the coefficient of variation (CV = standard deviation/mean) was calculated.

Phylogenetic generalized least squares (PGLS) analysis

Species data may not be free of phylogenetic association because shared character values may result from common ancestry rather than independent evolution, and thus may not be truly independent. To control for this phylogenetic inertia, we used phylogenetic generalized least squares (PGLS) analyses (Felsenstein 1985) to test for relationships between species differences in total and relative protamine and transition protein expression, and relative testes mass. PGLS analysis was implemented in COMPARE 4.6b (Martins 2004), using a phylogenetic tree based on Lundrigan et al (2002) and Gómez Montoto et al (2011a) (ANNEX IV Fig. S1).

Geometric morphometrics analysis of sperm head shape

Geometric morphometrics methods were used to quantify head shape variation based on a set of landmarks that correspond to the spatial position of particular anatomical traits (Kendall1986, Goodall 1991). A total of 20 bidimensional landmark coordinates were gathered from spermatozoa of seven of the eight species used in the gene expression analysis (*M. caroli, M. castaneus, M. domesticus, M. macedonicus, M. musculus, M. spicilegus, M. spretus*; n = 5 males/species). Landmark data were processed as described previously (Varea Sánchez et al 2013). All morphometric analyses were conducted with MorphoJ (Klingenberg 2011). An independent contrast for morphometric shape data (Klingenberg and Gidaszewski 2010) was conducted to check for phylogenetic signal in the sperm head shape data set. This test simulates the null hypothesis of total absence of phylogenetic signal by a permutation procedure. The P-value was not significant (P = 0.102) for the null hypothesis of independence, which indicates a lack of phylogenetic signal and, therefore, that phylogenetic correction was not needed for this analysis.

Canonical variate analysis (CVA) (Campbell and Atchley 1981) was used to explore the relationship between sperm head shape and relative protamine expression. Species were grouped into three categories based on well-defined differences in relative protamine expression: low, intermediate and high expression ratios (Table 1, ANNEX IV Table S2; see Results section for details). The CVA produces a set of canonical variates that are uncorrelated within and among groups and account for the maximum amount of among-group variance relative to within-group variance. As a result of the CVA, distances in the original space are transformed to Procrustes distances. These Procrustes distances for between-category comparisons were used to test for significant differences in sperm head shapes between species with low, intermediate and high protamine expression ratios.

RESULTS

Expression of protamines and transition nuclear proteins

Median expression levels for each gene and species are shown in table 1. The ranges of expression medians, and the coefficient of variation (CV) for each gene and species are provided in Table S2 (ANNEX IV). Within species, expression levels were positively correlated in all pairwise comparisons among genes (ANNEX IV Figure S2 and Table S3), suggesting that there may be functional constraint to maintain consistent relative expression levels of these genes and/or common regulatory control. The median expression level for individual genes varied by a factor of approximately threefold among species (ANNEX IV Table S2). *Tnp1* was expressed at a slightly higher level than *Tnp2* although both showed the same CV. Likewise, *Prm2* was expressed at a slightly higher level than *Prm1* but there was no difference in CV (Table 7.1, ANNEX IV Table S2).

The ratios and proportions of expression levels for different genes are shown in supplementary table S4. These relative levels of expression were much more constant among species (ANNEX IV Table S4) than expression levels of individual genes (cf. ANNEX IV Table S2). The ratio of total protamines to total transition nuclear proteins was close to one in half the species and above one in the other four species, revealing higher overall expression levels of protamines. Ratios between *Tnp1* and *Tnp2* were generally above one, in agreement with higher expression levels of *Tnp1* in comparison to *Tnp2* (see above). The reverse was true for protamines, with ratios of *Prm1/Prm2* below one (ANNEX IV Table S4).

Table 7.1. Relative testes mass was calculated as described by Kenagy and Trombulak (1986) followed by calculation of the median for the species. Gene expression data are normalized transformed median values. Species were ordered by relative testes mass (ascending).

species	relative testes mass	*Prm1* (ΔC_T)	*Prm2* (ΔC_T)	*Tnp1* (ΔC_T)	*Tnp2* (ΔC_T)	*Tnp1/ Tnp2*	*Prm1/ Prm2*	*Prm/ Tnp*	*Prm2/ Tnp*	*Prm2/ Prm*	*Prm2/ (Prm + Tnp)*
Mus castaneus	0.27	3.16	4.29	2.96	3.38	0.83	0.67	1.24	0.75	0.60	0.33
Mus pahari	0.27	3.80	3.69	3.01	2.26	1.13	1.01	1.38	0.68	0.50	0.29
Mus domesticus	0.32	2.11	3.22	1.88	2.32	0.81	0.65	1.27	0.77	0.61	0.34
Mus musculus	0.44	2.87	3.72	3.27	2.98	1.14	0.76	1.06	0.61	0.57	0.29
Mus caroli	0.46	5.83	7.28	6.71	6.18	1.07	0.78	1.00	0.56	0.56	0.28
Mus spretus	0.87	1.90	2.31	2.32	1.70	1.38	0.82	1.05	0.58	0.55	0.28
Mus macedonicus	0.95	3.54	3.49	2.48	2.14	1.05	0.99	1.49	0.74	0.50	0.30
Mus spicilegus	1.51	4.61	4.60	4.76	3.92	1.16	0.98	1.05	0.53	0.50	0.26
CV	0.69	0.37	0.36	0.46	0.46	0.18	0.17	0.15	0.14	0.08	0.09

Relationships between relative testes mass and gene expression

We tested for associations between relative testes mass and patterns of protamine and transition protein expression, both for individual genes and for ratios of expression levels among genes.

The correlation between relative testes mass and *Prm1/Prm2* or *Prm2/Prm* was not significant when all eight species were considered (Fig. 7.1A, Table 7.2). However, we noted that *M. pahari* appears to be an outlier in this analysis. *M. pahari* is basal to the other species included in this study and belongs to a different subgenus (*Coelomys*) (Veyrunes et al 2006). When the analysis was restricted to the seven species in the subgenus *Mus*, there was a significant positive relationship between relative testes mass and *Prm1/Prm2* (α = 15.5, CI 95% (slope) = 1.67 to 4.25, correlation = 0.89) (Fig. 7.1A, Table 7.2) and a significant negative relationship between relative testes mass and *Prm2/Prm* (α = 15.5, CI 95% (slope) = -0.14 to -0.06, correlation = 0.80) (Table 7.2). In contrast, there was no relationship between testes mass and transition protein ratios (data not shown).

Significant negative associations with relative testes mass were found for *Prm2/Tnp* (α = 1.56, CI 95% (slope) = -4.64 to -0.03, correlation = -0.63)(Fig. 7.1A, Table 7.2) and *Prm2/* (*Prm*+*Tnp*)(α = 6.05, CI 95% (slope) = -0.19 to -0.02, correlation = -0.72) (Table 7.2). In contrast, there was no association between relative testes mass and *Prm1/Tnp, Prm1/ (Prm+Tnp)* or *Prm/Tnp* (data not shown), or between relative testes mass and any of the four genes when analyzed separately (ANNEX IV Table S5). Thus, significant relationships between testes mass and the expression of sperm condensation proteins are driven mainly by the relative expression of *Prm2.*

Together, these results indicate that species with higher inferred levels of sperm competition express proportionately less *Prm2* in relation to total transition protein, and in relation to total protamine and transition protein combined. Within the subgenus *Mus*, species with higher sperm competition have a higher *Prm1/Prm2* expression ratio and therefore a lower *Prm2/Prm* proportion.

Relationships between protamine expression and sperm head shape

Geometric morphometrics was employed to quantify differences in head shape between the seven species in the subgenus *Mus*. Species were categorized as having high, intermediate or low protamine expression ratios, and Procrustes distances (*D*) calculated

from canonical variate analysis were used to test for between-category differences in sperm head shape.

Sperm head shapes were significantly different between species with high, intermediate and low *Prm1/Prm2* ratios (high vs. intermediate: $D = 0.08$, $P = 0.0002$; high vs. low: $D = 0.1$, $P = 0.0001$; intermediate vs. low: $D = 0.05$, $P = 0.05$; Fig. 7.2). The same between-category differences in sperm head shape were obtained for *Prm2/Prm* ratio (high vs. intermediate: $D = 0.05$, $P = 0.0001$; high vs. low: $D = 0.1$, $P = 0.0001$; intermediate vs. low: $D = 0.08$, $P = 0.0001$). These results support the idea that sperm head shape is influenced by relative protamine expression.

Table 7.2. Relationships between relative testes mass and relative protamine or transition protein expression. Analyses were carried out with all species and excluding *Mus pahari* (see text). CI- and CI+ indicate the confidence intervals for the regression slope, lnL = log likelihood estimate of alpha, alpha = measure of evolutionary constraints acting on phenotypes, corr = the correlation value (r). Bold CI values indicate statistical significance.

	excluding *Mus pahari* ($n = 7$)		relationships for all species ($n = 8$)			
	Prm1/Prm2	*Prm2/Prm*	*Prm2/Tnp*	*Prm2/(Prm + Tnp)*	*Prm1/Prm2*	*Prm2/Prm*
CI−	**1.67**	**−0.14**	**−4.64**	**−0.19**	−0.47	−0.12
CI+	**4.25**	**−0.06**	**−0.03**	**−0.02**	3.68	0.01
lnL	8.15	8.14	5.20	6.05	4.52	4.63
alpha	15.50	15.50	1.56	1.66	5.62	5.37
corr	0.89	−0.80	−0.63	−0.72	0.53	−0.54

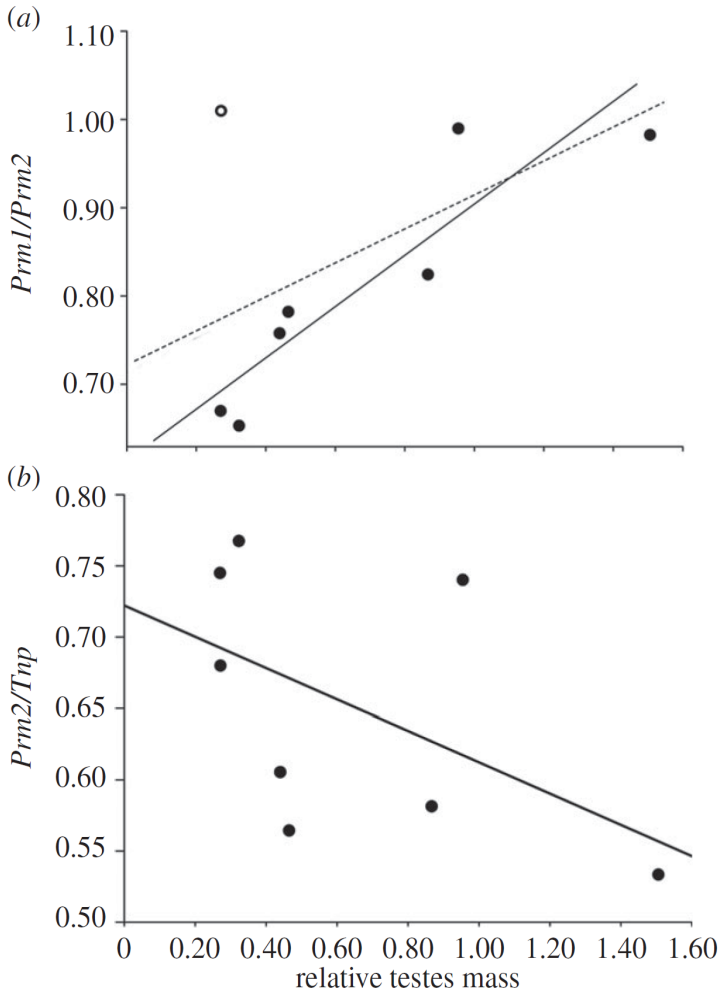

Figure 7.1. Relationships between relative testes mass and relative protamine 2 expression. (a) Protamine ratio (*Prm1/Prm2*): the dashed line corresponds to analyses with N = 8 mouse species, and the correlation is not significant. The open circle identifies *Mus pahari*, a species that behaves as an outlier in these analyses. The solid line corresponds to analyses with N = 7 species in which *M. pahari* is not included, and this correlation is statistically significant. (b) Ratio of *Prm2* to total *Tnp* (*Prm2/Tnp*). Results of statistical analyses are given in Table 2.

DISCUSSION

Despite the long-standing debate over the relative contribution of coding vs. regulatory changes to adaptive evolution (King and Wilson 1975, Carroll 2005, Hoekstra and Coyne 2007), mounting empirical evidence demonstrates that regulatory evolution can play a major role in adaptive divergence, particularly between closely related lineages (e.g. King and Wilson 1975, Carleton and Kocher 2001, Shapiro et al 2004, Abzhanov et al 2004, 2006, Manceau et al 2011, Jones et al 2012). In this study we compared protamine and transition nuclear protein mRNA expression in the testes of eight species in the genus *Mus* that share recent common ancestry but differ widely in inferred levels of sperm competition. We found that species that experience higher levels of sperm competition express less *protamine 2* in relation to both transition nuclear proteins, and to *protamine 1*. This strongly suggests that species differences in relative expression levels of these key spermiogenesis genes are influenced by variation in the strength of post-copulatory sexual selection. The fact that this pattern is driven by the relative expression of *protamine 2* is consistent with evidence that the promoter region of this gene is evolving under sexual selection in *Mus* (Martin-Coello et al 2009). Importantly, we found that species that differ in ratios of *protamine 2* expression, both in relation to *protamine 1* and in relation to total protamines, also differ in sperm head shape. This suggests that regulatory changes contribute to modifications of sperm phenotype that could, ultimately, influence sperm's competitive ability. Taken together, the results of this study support the proposition that selection on regulatory regions can fine-tune adaptive phenotypes on short evolutionary timescales (Wray 2007). We discuss these results in relation to previous work on the evolution of sperm chromatin condensation genes in mammals, and the genetics and functional consequences of sperm competition in rodents.

Prm1/Prm2 ratio		high	intermediate	low
				
Prm1/Prm2 ratio	range	0.98 – 0.99	0.76 – 0.82	0.65 – 0.67
	intermediate	$D = 0.08$ $p = 0.0002$	—	—
	low	$D = 0.10$ $p = 0.0001$	$D = 0.05$ $p = 0.05$	—

Figure 7.2. Procrustes distances (d) and P values for canonical variate analyses examining head shape in relation to Prm1/Prm2 ratio. Three groups of species were defined according to their ratios of protamine expression: high (M. macedonicus, M. spicilegus), intermediate (M. musculus, M. caroli, M. spretus) and low (M. castaneus, M. domesticus)(see Table 1). Morphometric data were taken from 35 individuals of 7 species. Procrustes distances different from zero indicate shape differences between groups. Wireframe graphics show the shape associated to each group categorized according to its Prm1/Prm2 ratio.

Protamines and sperm competition: evolution at two levels

Sperm chromatin condensation genes, including protamines, are thought to be among the fastest evolving male reproductive proteins in eutherian mammals (e.g. Queralt et al 1995, Su et al 2013). There is ample evidence from primates and rodents that selection contributes to this rapid rate of change (Rooney and Zhang 1999, Wyckoff et al 2000, Torgerson et al 2002, Turner and Hoekstra 2008) and sperm competition is often invoked as the driving force (Swanson and Vacquier 2002). However, how particular substitutions might enhance sperm competitiveness remains untested, and it has been suggested that selection for protein stability is an equally parsimonious explanation for protamine coding sequence evolution in primates (Clark and Civetta 2000). Notably, in case-control studies of human males, associations between infertility and coding region SNPs in either *Prm1* or *Prm2* are rare (e.g. Schlicker et al 1994, Aoki et al 2006, He et al 2012), whereas men with imbalanced PRM1/PRM2 ratios are consistently subfertile or sterile (reviewed in Carrell et al 2007). Thus, while the functional consequences of protamine coding sequence substitutions are largely unknown, changes in protamine expression have a demonstrated impact on male fertility, and therefore might co-vary with the strength of post-copulatory sexual selection across species.

In the *Mus* clade comprising house mice and their close relatives, there is evidence for weak positive selection on *Prm2* coding sequence in the three species with the highest inferred levels of sperm competition (*M. spicilegus, M. spretus* and *M. macedonicus*), whereas divergence in the promoter region is positively correlated with relative testes mass, and with sperm swimming speed, across the entire clade (Martin-Coello et al 2009). Here, using a subset of the same species, we show that the relative abundance of *Prm2* mRNA in the testes is negatively correlated with relative testes mass. These findings suggest that nucleotide substitutions in the *Prm2* promoter region influence expression, and that high levels of sperm competition act to decrease the relative abundance of *Prm2* in the testes.

We emphasize, however, that our understanding of the relationship between protamine 2 regulation and sperm competition in *Mus* is far from complete. First, the functional relationship between promoter evolution and expression is not straightforward: species with higher *Prm2* promoter divergence express less *Prm2* only in relation to transition nuclear proteins and *Prm1*. Despite substantial interspecific differences in the expression levels of all four genes, there was no relationship between relative testes mass and individual gene expression. Likewise, although the *Prm1* promoter region is highly variable in *Mus*, there is no re-

lationship between divergence and levels of sperm competition (Martin-Coello et al 2009). A plausible explanation for these patterns is that sexual selection for reduced PRM2 is counter-balanced by natural selection to maintain the relative proportions of protamines and transition nuclear proteins within a functional range. Potential mechanisms include compensatory evolution in the promoter regions of interacting sperm chromatin condensation proteins, or a single regulatory modifier shared among genes. In mice, as in humans, *Prm1, Prm2* and *Tnp2* are tightly clustered in the genome. Thus, an enhancer element common to all three genes is a formal possibility. Comparative analysis of intergenic regions in the *Prm1/Prm2/Tnp2* cluster, together with the *Tnp1* and *Tnp2* promoter regions, will help to discriminate these non-mutually exclusive alternatives.

Second, the correlation between mRNA expression levels and protein abundance is often imperfect (Greenbaum et al 2003). Quantification of sperm chromatin condensation proteins in mature spermatozoa will provide a direct measure of species differences in their relative abundance. Finally, evidence for selection on *Prm2* coding sequence in *M. spicilegus, M. spretus* and *M. macedonicus* is intriguing, because it suggests that high levels of sperm competition can drive coding and regulatory evolution in tandem (Martin-Coello et al 2009). However, whether positively-selected *Prm2* amino acid substitutions in these species affect sperm phenotypes related to competitive ability remains to be determined.

The relative abundance of protamine 2: functional implications for sperm phenotypes

Why should high levels of sperm competition favour reduction in the relative abundance of PRM2? While the phenotypic effects of inter-specific differences in protamine ratios are largely unstudied, there is some evidence that sperm from species that either lack PRM2, or produce very little PRM2 relative to PRM1, exhibit slower DNA decondensation in the oocyte (Perreault et al 1988, Corzett et al 2002). Sperm with more compact heads may have higher competitive ability (Tourmente et al 2011a), and sperm with incomplete DNA compaction often have over-sized or less streamlined heads (Balhorn 2007). Thus, it is plausible that high levels of sperm competition select for higher DNA compaction, and thus proportionately less PRM2. Evaluation of this hypothesis will require comparative analyses of sperm chromatin compaction in relation to head morphology, the proportion of PRM2, and the strength of sexual selection mediated by sperm competition. Notably, the finding that relative abundance of *Prm2* is associated with differences in sperm head shape is an

important first step towards revealing the functional relationship between protamine expression and sperm head morphology. Future studies will investigate the hydrodynamic consequences of these *Prm2*-associated differences in sperm head shape.

Conclusions

An important role of comparative studies such as this is to identify patterns that generate testable hypotheses (Harvey and Pagel 1991). Here, we show that species of mice with higher inferred levels of sperm competition express less protamine *2* in relation to *protamine 1* and transition nuclear proteins. Based on this pattern, together with evidence for sexually selected divergence in the promoter region of protamine *2* (Martin-Coello et al 2009), we propose that reduction in the relative abundance of protamine *2* enhances sperm competitive ability in mice by influencing sperm head shape, and that regulatory evolution plays a key role in this evolutionarily rapid response to selection.

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Lüke L, Campbell P, Varea-Sánchez M, Nachman MW, Roldan ERS (2014) Sexual selection on protamine and transition nuclear protein expression in mouse species. *Proceedings of the Royal Society of London, series B* 281: 20133359. doi: 10.1098/rspb.2013.3359.

CHAPTER 8

PROTAMINES AND SPERM HEAD PHENOTYPE: A COMPARATIVE ANALYSIS

RESUMEN

La selección sexual postcópula influye en la evolución de los genes reproductivos, en la regulación de la expresión génica y en la forma y función de los espermatozoides en los mamíferos. En este trabajo investigamos las diferencias en la expresión de protaminas y sus efectos en la forma y dimensiones de la cabeza del espermatozoide de dos familias de roedores, múridos y cricétidos. Hemos examinado el efecto de la selección sexual postcópula en los niveles de expresión y fenotipo de los espermatozides. En topillos (cricétidos), a pesar de un nivel de expresión de *Pm1* comparativamente mayor, los ratios menores de expresión de protaminas (*Pm1/Pm2*), y un menor nivel de expresión absoluta de protaminas parecen ser favorecidas en especies con mayores nivels de competición espermática. Además, la presión selectiva que afecta la expresión de protaminas en topillos parece estar asociada con un menor tamaño relativo de la cabeza del espermatozoide. Se observa un patrón opuesto en ratones (múridos). En este grupo, se ve favorecido un ratio mayor de expresión de protaminas, aparentemente por la reducción relativa de expresión de *Pm2*, en especies con alta competición espermática. La presión selectiva que afecta los ratios de protamina se asocia con un aumento relativo del tamaño de la cabeza espermática. Hemos encontrado diferencias esenciales en la manera en que las protaminas son seleccionadas e intervienen en la estructura fenotípica de la cabeza del espermatozoide en topillos y ratones. Una reducción relativa de la expresión de *Pm2*, y el subsiguiente incremento en el ratio de protaminas, parecen ser el principal punto de acción de la selección sexual en ratones. Mientras el ratio de protaminas tiene un efecto en el fenotipo de la cabeza espermática en topillos, un incremento en los niveles de expresión de ambas protaminas parece ser el principal punto de acción de la selección sexual.

SUMMARY

Postcopulatory sexual selection is known to influence the evolution of the so-called reproductive genes, regulation of gene expression, and sperm form and function in mammals. Here we investigate the differences in protamine expression and their effects on sperm head form and dimensions in two rodent families, murids and cricetids. We examined the effect of postcopulatory sexual selection on expression levels and, in turn, on sperm head phenotype. In voles (cricetid species), despite a comparatively higher *Prm1* expression level, lower protamine expression ratios (*Prm*1/*Prm*2), and lower absolute protamine expression levels seem to be favoured in species with high levels of sperm competition. In addition, selective pressure affecting protamine expression in voles seems to be associated with smaller relative sperm head size. The opposite pattern was observed in mice (murid species). In this group, an increase of the protamine expression ratio, seemingly by a relative reduction of *Prm2* expression, is favoured in highly competitive species. Selective pressure affecting the protamine ratio associates with an increase in relative sperm head size. We found essential differences in the way protamines are selected and involved in shaping sperm head phenotype in voles and mice. A relative reduction of *Prm2* expression, and therefore an increase in protamine ratio, seems to be the main target of sexual selection in mice. While the protamine ratio does have an effect on sperm head phenotype in voles, an increase of the expression levels of both protamines seems to be the main target of sexual selection.

INTRODUCTION

The evolution of phenotype through changes in genotype is the subject of great interest. Many studies have addressed this issue in an effort to understand the processes of development and evolution but, also, the genetic basis of disease. In order to clarify the function and phenotypic effect of a gene the usual approach is reverse genetics. Candidate gene products are disrupted or modified (e.g., over-expressed, knocked-out) followed by an evaluation of the resulting phenotypic changes. Although this is the most direct strategy it has the disadvantage of exaggerating the phenotype resulting, in many cases, in non functionality and false positives (Gilchrist and Haughn 2005). However knowledge on genotype-phenotype associations can be gained by carefully comparing and interpreting naturally occurring diversity and selective pressures. This approach allows for a more fine-tuned evaluation of phenotype, as affected by genotype diversity and the selective pressure by which it is defined, as opposed to that observed by genetic modification or experimental evolution.

The study presented here is an example of this approach. We aimed to analyze the relationship between protamine regulatory sequences and expression diversity on sperm head phenotype in two rodent families. We observed that the relationship between protamines and sperm phenotype is not straightforward and depends on a multitude of factors involved in sperm function and competition. We purport to illustrate that interpreting genotype-phenotype associations through a correlational approach, in a comparative evolutionary model, allows for a more precise interpretation of a highly complex relationship.

What drives regulatory evolution or changes in gene coding sequences? Among genes that regulate reproductive function, it has been proposed that sperm competition (a form of postcopulatory sexual selection) is an evolutionary process that acts on sperm of rival males that compete for the fertilization of ova (Parker 1970). This selective force drives the adaptation of sperm function and morphology (reviewed in Birkhead and Moller 1998, Simmons 2001, Birkhead et al 2009). Understanding how sperm competition can shape phenotypes through changes in gene and regulatory DNA sequences allows us to draw conclusions about evolutionary processes in general and the evolution of specific function.

Mammals have evolved a great diversity of male gamete morphologies which is thought to be mainly driven by postcopulatory sexual selection, specifically sperm competition. Rodents show a wide range of sperm competition levels within a broad range of species

and a particular diversity in sperm phenotypes (Cummins and Woodall 1985, Roldan et al 1992, Pitnick et al 2009).

Protamines are basic arginine rich sperm nuclear proteins involved in the complete reorganization of sperm cell chromatin which is taking place during sperm development (Oliva and Dixon 1991). During this process the entire spermatid genome is globally inactivated, highly condensed and protected. The role of protamines, however, does not end here. When binding to the DNA, condensing it into tight coiled structures, enough force is produced to reduce the entire sperm nucleus to a small volume and even affect sperm head shape (Balhorn and Balhorn 2011). Previous studies on the effect of protamines on phenotype have been done mainly concentrating on humans and rodents searching for explanations of male idiopathic infertility (Aoki et al 2005, Oliva et al 2006, Jodar et al 2010). These studies have demonstrated that abnormal protamine expression results in altered sperm head morphologies and infertility (Cho et al 2001, Aoki et al 2005). Incorrect chromatin condensation leads to abnormal sperm head morphologies as well as larger sperm heads in general (Belokopytova et al 1993). Both protamines have been demonstrated to be essential for male fertility, while the ratio between protamine 1 and 2 seems to be of specific importance (de Yebra et al 1998, Carrell et al 2001). The protamine ratio seems to be stable within a species but highly varies among mammals (Corzett et al 2002).

Other studies approached the question based on a comparative evolutionary approach. A relationship between changes in protamine coding sequence and sperm head size was found in voles and hamsters (Lüke et al 2014a). A higher evolutionary rate in the coding sequences of both protamines associate with an increase in relative head size and a decrease in head elongation while a smaller and more elongated head seems to be generally favoured in cricetid species with higher levels of sperm competition (Lüke et al 2014a). A study on protamine and transition protamine expression in mice revealed that sexual selection seems to reduce the relative amount of *Prm2* in the expression ratio of protamines. This study also reported the first evidence for the effect of protamine expression on sperm head shape. Different protamine ratios associate clearly with significantly different head shapes (Lüke et al 2014b). The connection between protamines and sperm head morphology is therefore already well supported. However, how and to what extent protamines affect sperm head shape remains to be elucidated.

We examined two rodent subfamilies (mice and voles) exhibiting a similar range in sperm competition levels but marked differences in the sperm head morphology between the

groups (Varea Sánchez 2014). The comparison of these two groups allowed us to carry out analyses of the relationship between protamine regulatory and expression divergence and sperm phenotype divergence driven by the differences in selective pressures through sperm competition. Eight species of the genus *Mus* were included as a group of closely related species with recent divergence (Guénet and Bonhomme 2003). On the other hand, seven species of *Arvicolinae* were included to represent a more diverse group of species with higher sequence divergence than the *Mus* group (Steppan et al 2004).

We predicted differences in protamine expression, structure, and selective pressure between groups, since sperm head morphologies show marked differences. We anticipated that sperm competition would affect the divergence of protamine regulatory sequences which, in turn, would have an effect on protamine gene expression. Since the protamine expression ratio has been demonstrated to be of vital importance for fertility, being under selective pressure through sperm competition and having an effect on sperm head shape, the protamine expression ratio should be of importance and would affect sperm head shape in both mice and voles, although perhaps in different ways.

MATERIAL AND METHODS

Species

This study included 8 species of the genus *Mus* in the group of mice: *M. caroli, M. castaneus, M. domesticus, M. macedonicus, M. musculus, M. pahari, M. spicilegus, and M. spretus* (4 to 5 males per species) and 7 species of the subfamily *Arvicolinae* in the group of voles: *Arvicola sapidus, Arvicola terrestris, Myodes glareolus, Chinomys nivalis, Microtus arvalis, Microtus cabrerae, Microtus duodecimostatus* (4 to 6 males per species). These two groups of species show diverse levels of sperm competition in comparable ranges. Large testes in relation to body mass (relative testes mass) is a strong predictor of high sperm competition levels in many taxa (reviewed in Kenagy and Trombulak 1986, Birkhead and Moeller 1998, Birkhead et al 2009), and relative testes mass is correlated with genetic paternity (i.e., percentages of multiple paternity) in mammals in general (Gomendio et al 1998), and rodents in particular (Soulsbury 2010). Therefore, relative testes mass is used in this study as a proxy for sperm competition levels. For comparison of relative testes mass between species we used residual testes mass taken from multiple regession analysis of body mass (log) and testes mass (log) (hereafter “residual testes

mass") (Table 1). For regression analysis between relative testes mass and other variables we included both body mass (log) and testes mass (log) as independent values in a phylogenetically corrected multiple regression (hereafter "relative testes mass").

Individuals belonging to the genus *Mus* were purchased from the Institut des Sciences de l'Evolution-Montpellier, CNRS-Universite de Montpellier II. Individuals belonging to *Arvicolinae* were trapped in the field during the breeding season at different locations in Spain (Gomez Montoto et al 2011). Males were kept in our animal facilities in individual cages under standard laboratory conditions in environmentally-controlled rooms (20 - 24ºC) on a 14 h light - 10 h darkness photoperiod, and were provided with food and water ad libitum. All animal handling was done following Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2010/63.

Testes collection and sperm measurements

Animals were sacrificed at an age of 2 to 4 months by cervical dislocation and were immediately weighed and dissected. Testes were removed, weighed, flash-frozen in liquid nitrogen, and stored at -80ºC. All dissection instruments and areas were cleaned with RNase AWAY® (Molecular BioProducts, Thermo Fisher Scientific, San Diego, CA) before use. Mature sperm were collected from both epididymides and vasa deferentia as described (Gómez Montoto 2011) and suspended in a Hepes-buffered modified Tyrode's medium (mT-H) under air (Shi and Roldan 1995). Sperm dimensions were measured in sperm smears stained first with eosin-nigrosin and subsequently with Giemsa as described previously (Gómez Montoto 2011). Spermatozoa were examined at 1000x under bright field and 200 sperm cells per male were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

DNA Isolation and promoter amplification

Protamine promoter sequences of Mus species were taken from NCBI Genbank (Martin-Coello et al 2009). Genomic DNA of *Arvicolinae* species was extracted from frozen tissues using the E.Z.N.A® Tissue DNA kit (Omega, Madrid, Spain) following the manufacturer's recommendations. Protamine promoter sequences were amplified by gene walking polymerase chain reaction (PCR) using the APAgene™ GOLD Genome Walking Kit (RT) following the manufacturers recommendations. All PCRs were performed in a Veriti thermocycler (Applied-Biosystems). PCR primers were designed on the basis of protamine genomic sequences available in NCBI Genbank and previous studies (Lüke et al 2011)

following the recommendations APAgene™ GOLD Genome Walking Kit (RT) protocol. All alignments were performed in Geneious R7 (created by Biomatters, available from http://www.geneious.com/). Specific bands were obtained after separation in a 1.5% agarose gel extracted with E.Z.N.A.® Gel Extraction Kit (Omega). Purified products were sequenced (Secugen S.L., Madrid, Spain).

Promoter sequence divergence rates

Promoter sequence divergence was determined using the program BaseML implemented in PAML 4. BaseML allows for calculation of nucleotide substitution rates as a measure of sequence divergence for branches of a given alignment and phylogenetic tree and for calculation of nucleotide substitution rate variation among sites of an alignment. Nucleotide subsitution model was determined by JModelTest (Protamine 1 promoter (ProP1): HKY, *Prm2* promoter (ProP2): JC). The association between morphometric and genetic data demands the calculation of sequence divergence rates that take into account not only selective pressure acting on the terminal branch but the accumulated selective pressure on the sequence during its evolution to the tip of the branch (root-to-tip divergence rate) in the selected group of taxa. Calculating sequence divergence this way, values obtained become more comparable with measured phenotypical data since the latter also represents the accumulated evolution rather than being the result of changes solely on the terminal branch (Montgomery et al 2011, Lüke et al 2011, 2014a).

RNA extraction and cDNA synthesis

RNA was extracted in a sterile vertical laminar flow hood using either the RNeasy Plus kit (Qiagen) or the E.Z.N.A® Total RNA kit I (Omega, Madrid, Spain) following the manufacturer's recommendations. All instruments and surface areas were cleaned with RNase AWAY®. RNA concentration and purity were determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Madrid, Spain) and cDNA was synthesized the same day from 10 µg of RNA, using the Superscript III First Strand Synthesis Kit with oligo(dT) (Invitrogen, Barcelona, Spain) according to the manufacturer's recommendations. cDNA concentration and purity were determined using a NanoDrop 1000 spectrophotometer and samples were stored at -20ºC.

Quantitative PCR (qPCR)

Expression levels for *Mus* species were available from previous studies (Lüke et al 2014b). Expression levels for *Arvicolinae* species were determined using a CFX96 Real Time System / C1000 Thermal Cycler (Bio-Rad). Primers were designed in Primer3 (v. 0.4.0) to amplify a product between 70 and 150 bases across an exon-exon junction. Protamine primers were placed in sequences that are invariant across all species in this analysis. Primer sequences are provided in ANNEX V Table S1. Each qPCR run included one individual of each species with three technical replicates for the two experimental genes (*Prm1, Prm2*), and two technical replicates for the standard gene (*18SrRNA*). qPCR reactions were run in 96-well plates with an end volume of 16 µl per sample containing 8 µl SYBR green Master Mix (Invitrogen), 15 ng of each primer and 50 ng/µl of cDNA. The conditions of the thermocycler program consisted of an initial denaturation of 95ºC for 10 min, 40 cycles of 95ºC for 15 sec and an annealing and elongation stage of 62ºC for 1 min. Melt curve analysis was performed at the end of each run to check for multiple peaks, indicative of non-specific amplification.

Analysis of expression data

Cycle threshold data (CT) were normalized relative to 18SrRNA for each plate (ΔCT). To avoid statistical analysis using a dataset of mixed negative and positive values, data were transformed by adding a constant based on the lowest ΔCT value in the joined dataset (*Mus* and *Arvicolinae*). Expression ratios and percentages were calculated from transformed individual ΔCT values (between 4 and 5 individuals per species), and mean values were obtained for each species. Because of the expectation that relative expression levels may be of greater functional significance than absolute expression levels (see above) the protamine ratio was calculated (*Prm1/Prm2*).

Phylogenetic Generalized Least Squares (PGLS) Analysis

Species data may not be free of phylogenetic association because shared character values may result from common ancestry rather than independent evolution, and thus may not be truly independent. To control for this phylogenetic inertia, we used phylogenetic generalized least squares (PGLS) analyses (Felsenstein 1985) to test for relationships between species differences in total and relative protamine expression, and relative testes mass. PGLS analysis was performed using the R-package Caper, using a phylogenetic tree based on Fabre et al (2012) (ANNEX V Fig. S1). To account for the effect of body

mass on testes mass we included both body mass and testes mass as independent values in a PGLS regression (relative testes mass) which is used as a proxy for sperm competition.

RESULTS

Differences between mice and voles

(a) Protamine promoter divergence. Unlike the *Mus* promoter sequences (Martin-Coello et al 2009) the promoter sequence of *Prm2* (*Prm2* promoter) of *Arvicolinae* was highly variable and not alignable beyond 250 bases upstream of the *Prm2* coding sequence. We therefore chose to concentrate analyses of the *Prm2* promoter alignment to 250 bases for both groups to ensure proper comparisons. Species promoter divergence rates and mean values are shown in Table 1. Divergence rates observed for the sequence of the protamine 1 promoter (*Prm1* promoter) were significantly higher in voles than in mice (t_{12}=5.5, p<0.001), while no significant difference was found for the *Prm2* promoter between these two groups. The *Prm1* promoter showed more sequence divergence than the *Prm2* promoter in both mice ($t_{8.48}$=3.58, p<0.05) and voles ($t_{12.57}$=7.76, p<0.001).

***(b) Protamine expression*.** Expression levels and mean values for the different species are presented in Table 8.1. A comparison of expression levels between the two groups revealed that voles express more protamine 1 (*Prm1*) than mice ($t_{12.81}$=8.52, p<0.001) while these groups do not differ significantly in their protamine 2 (*Prm2*) expression levels ($t_{9.22}$=2.1, p=0.06). As a consequence, voles show a significantly higher protamine ratio than mice ($t_{7.2}$=4.5, p<0.05). Voles express significantly more *Prm1* than *Prm2* ($t_{8.99}$=2.62, p<0.05), while this difference was not found in mice ($t_{13.93}$=-1.04, p=0.31).

***(c) Sperm head size*.** Because total sperm length varies greatly among mouse and vole species, and drag resulting from head size should be analyzed taking into account the length of the flagellum (Humphries et al 2008), sperm head dimensions were calculated as proportions of total sperm length (hereafter, relative head lengh, relative head width, relative head area). Significant differences in relative head width were found between voles and mice ($t_{8.43}$=12.42, p<0.05). Sperm heads of voles appear to be wider. No such differences could be found for relative head length ($t_{11.01}$=0.62, p=0.55) or area ($t_{10.54}$=-0.24, p=0.81). Data and mean values of relative head dimensions for the different species are given in Table 8.1.

Table 8.1. Summary of residual testes mass and protamine genotype data. Relative testes mass was taken from regression analysis using species mean testes mass as dependent and species mean body mass as independent value. Gene expression data are normalized, transformed mean values. Sperm head size measurements are calculated relative to total sperm length. Differences between the two taxa were tested via Welsch two sample t-test, asterisks indicate statistical significance (* = p<0.05, ** =p<0.001). Species ordered by residual testes mass (ascending).

Species	residual testes mass	Expression data			Promoter divergence		Sperm head dimensions (µm)		
		*Prm*1 (ΔC_T)	*Prm*2 (ΔC_T)	*Prm1/ Prm2*	*Prm1 Promoter*	*Prm2 Promoter*	Relative sperm head width	Relative sperm head length	Relative sperm head area
Microtus duodecimcostatus	−0.595	22.750	19.695	1.159	0.154	0.085	0.051	0.081	0.205
Microtus cabrerae	−0.546	23.105	20.223	1.143	0.166	0.060	0.050	0.076	0.238
Arvicola terrestris	−0.447	22.090	16.876	1.311	0.166	0.055	0.028	0.060	0.154
Arvicola sapidus	−0.157	22.774	19.719	1.154	0.171	0.041	0.035	0.061	0.184
Microtus arvalis	−0.048	22.718	22.477	1.011	0.173	0.085	0.037	0.075	0.214
Myodes glareolus	0.201	25.592	23.705	1.079	0.108	0.026	0.045	0.081	0.257
Chionomys nivalis	0.223	24.254	22.490	1.079	0.108	0.059	0.044	0.080	0.288
mean:	−0.195	23.326	20.741	1.134	0.149	0.059	0.041	0.073	0.220
Mus pahari	−0.459	18.647	18.662	0.999	0.085	0.092	0.037	0.070	0.283
Mus castaneus	−0.394	17.221	18.495	0.931	0.087	0.070	0.027	0.062	0.180
Mus domesticus	−0.320	16.541	17.684	0.935	0.096	0.065	0.028	0.064	0.191
Mus musculus	−0.209	17.444	18.333	0.952	0.090	0.065	0.032	0.075	0.233
Mus caroli	−0.099	19.914	21.463	0.928			0.034	0.065	0.223
Mus macedonicus	0.164	18.251	18.234	1.001	0.082	0.080	0.035	0.075	0.227
Mus spretus	0.218	16.718	17.093	0.978	0.091	0.065	0.038	0.073	0.238
Mus spicilegus	0.374	19.265	19.350	0.996	0.086	0.075	0.033	0.082	0.223
mean:	−0.091	18.000	18.664	0.965	0.088	0.073	0.033	0.071	0.225
difference between groups:		**		*	**		*		

(f) Residual testes mass, a proxy of sperm competition levels. The association of relative testes mass to levels of sperm competition in many taxa (Birkhead and Moller 1998, Gomendio et al. 1998, Birkhead et al 2009) and its relation to levels of genetic paternity (Soulsbury and Dornhaus 2010) makes relative testes mass a widely used and very reliable proxy for level of sperm competition. For comparison of relative testes mass between species we used residual testes mass taken from multiple regession analysis of body mass (log) and testes mass (log) (herafter: residual testes mass). For regression analysis between relative testes mass and other variables we included both body mass (log) and testes mass (log) as independent values in a phylogenetically corrected multiple regression (hereafter: relative testes mass). The two groups of species exhibited high diversity but similar ranges of residual testes mass (mice: range = -0.6 to 0.22, mean = -0.2; voles: range = -0.46 to 0.37, mean = -0.1) (Table 8.1). Consequently these groups are adequate for an comparative evolutionary study based on sperm competition as driving force.

Sperm competition, protamines and sperm head morphology

(a) Rationale. In order to test for associations between sperm competition, protamine gene expression, its regulatory sequences and sperm head morphology we performed phylogenetic generalized least squares regression analyses (PGLS) (see Material and Methods). We analyzed several hypothetical associations (Fig. 8.1): According to previous studies (Martin-Coello et al 2009, Lüke et al 2014a,b) we expected the sperm competition level to affect protamine promoter sequence divergence, protamine expression as well as sperm head morphology. We anticipated that changes in protamine promoter sequences would have an effect on protamine expression levels. And, finally, we expected protamine expression levels to be related to sperm head morphology. This study focusses of the effect of protamine expression on sperm head dimensions. Sperm head shape data (geometric morphometric data) were not used due to lack of statistical power with the current sample size. Figure 8.2 summarizes the results of the genotype-phenotype association analysis; details are given below. Detailed PGLS results are presented in ANNEX V Table S2.

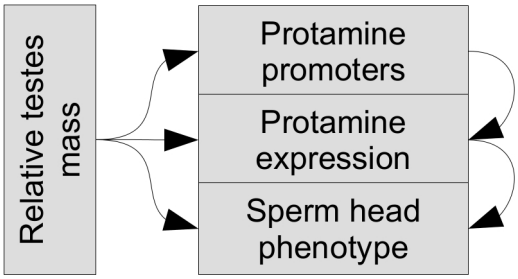


Figure 8.1. Hypothetical associations between protamines genotype and sperm head phenotype.

(b) Effect of sperm competition on protamines. Among mice we found a positive correlation of relative testes mass with protamine expression ratio. No significant relationships were found between relative testes mass and promoter sequence divergence in these species. For voles we found a negative correlation between relative testes mass and *Prm1* promoter divergence as well as a trend for a negative correlation between relative testes mass and the protamine ratio. A significant positive relationship was found between relative testes mass and both *Prm1* and *Prm2* expression levels. Detailed PGLS results are presented in ANNEX V Table S2.

(c) Effect of promoters on protamine expression. For mice we found a negative correlation between *Prm1* promoter divergence and *Prm1* expression levels. In contrast *Prm2* promoter divergence rate shows a significant positive effect on *Prm1* expression

levels. No effect of promoter divergence rate on *Prm2* expression levels was found. For voles we found a negative correlation of *Prm1* promoter divergence on both *Prm1* and *Prm2* expression levels. No effect of *Prm2* promoter divergence on protamine expression was found for voles. Detailed PGLS results are presented in ANNEX V Table S2.

(d) Effects on sperm head size. Among mouse species we found a positive relationship between relative testes mass and relative sperm head length and width, as well as a positive trend for relative sperm head area. Higher levels of sperm competition thus seem to result in selective pressure to increase sperm head size in this group of species. The protamine expression ratio showed similar effects on sperm head size in mice: a positive association was found between the protamine expression ratio and relative sperm head width and area, and a clear trend for relative head length.

For vole species no direct association of relative testes mass and sperm head dimensions was found. The protamine expression ratio showed a negative association with relative head length and area, while both total *Prm1* and *Prm2* expression showed positive association with relative head length and area. An indirect effect of relative testes mass on sperm head dimensions seems likely to occur because of the negative associations of relative testes mass with the *Prm1* promoter and *Prm1* and *Prm2* expression, as well as the negative trend for protamine ratio. Detailed PGLS results are presented in ANNEX V Table S2.

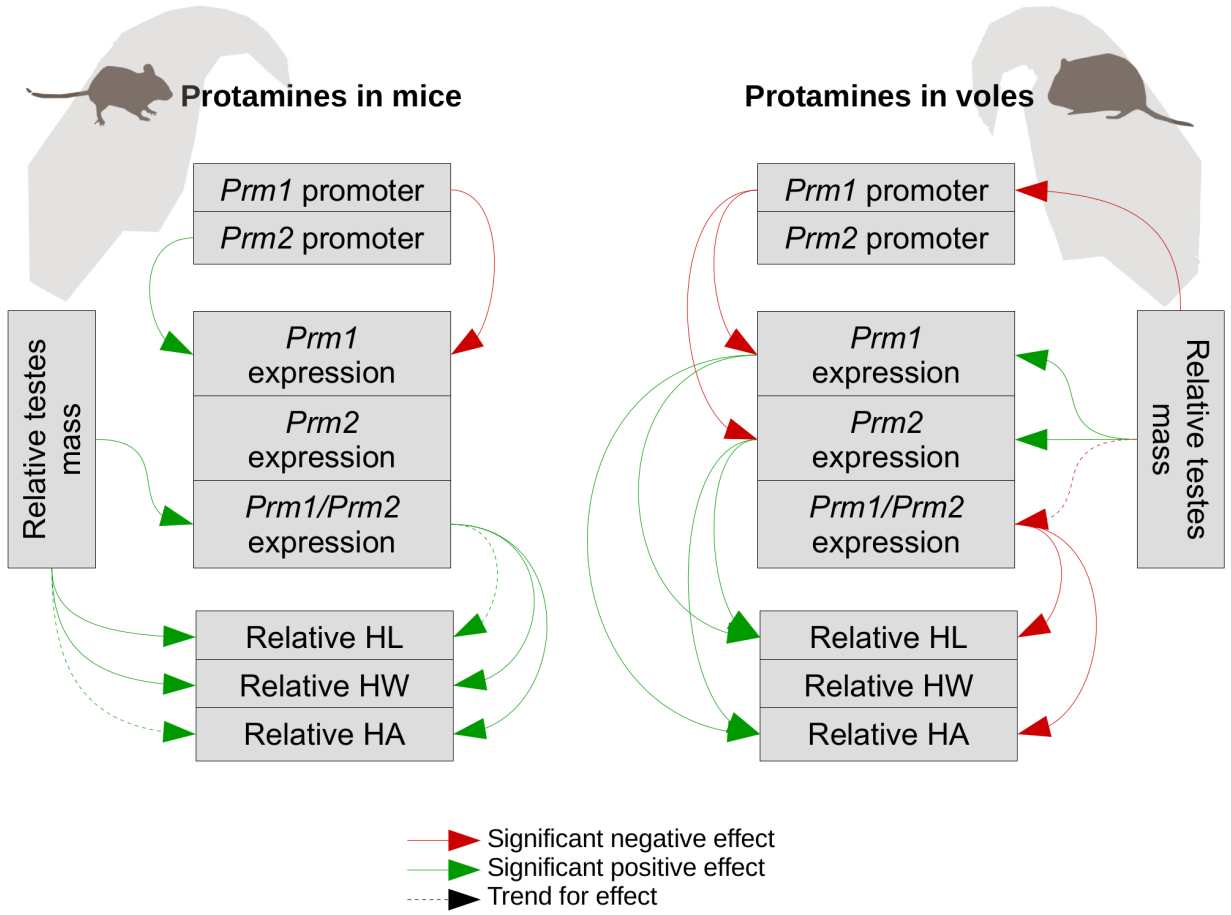


Figure 8.2. Representation of PGLS results comparing mice and voles. HL = sperm head length, HW = sperm head width, HA = sperm head area.

DISCUSSION

We found essential differences in the way protamines are selected and utilized to shape sperm head phenotype in voles and mice. In mice, an increase of the protamine ratio, seemingly by relative reduction of *Prm2* expression, is favoured in highly competitive species. Selective pressure affecting the protamine ratio seems to result in an increase in relative sperm head size. The opposite pattern was observed in voles. Despite a comparatively higher *Prm1* expression level, lower protamine expression ratios (*Prm*1/*Prm*2), and lower absolute protamine expression levels seem to be favoured in species with high levels of sperm competition. In this group, selective pressure affecting protamine expression seems to result in smaller relative sperm head size.

Protamines in the complex interplay between the sperm cell and fertilization environment

It is possible that protamines may be affected by various types of selection resulting in an equilibrium between conservation of function and adaptations to high sperm competition levels. In mammals protamines are diverse with highly conserved regions (Oliva and Dixon 1991, Rooney et al 2000, Wyckoff et al 2000). In primates the coding sequence of *PRM1* has been shown to be positively selected (Wyckoff et al 2000). However, within rodents, *Prm1* has been shown to be functionally conserved with directed positive selection on a few C-terminal sites, while *Prm2* is under relaxed constraint (Lüke et al 2011). Under the pressure of sperm competition protamines might impact on sperm head phenotype to become more streamlined thus increasing sperm velocity. However, which type of sperm head is the most hydrodynamically efficient is not known. The complex interaction between sperm metabolism, flagellar beating pattern, sperm size, hook and nucleus size and shape has to be taken into account in order to evaluate the advantages of a certain sperm head phenotype for sperm swimming velocity (Malo et al 2006, Gómez-Montoto et al 2011, Tourmente et al 2013). In this study we focus on associations with sperm head dimensions. Sperm head shape data (geometric morphometric data) were not used due to lack of statistical power with the current sample size.

Sexual selection on regulation of protamine expression

In both mice and voles we found evidence for an effect of protamine promoter divergence rates on protamine expression levels. However, an effect of sexual selection (sperm

competition) on protamine promoter divergence was only found for the *Prm1* promoter in voles. A previous study by Martin-Coello et al (2009) described an effect of sexual selection in the form of sperm competition on *Prm2* promoter divergence in mice. Unlike the *Mus* promoter sequences (Martin-Coello et al 2009) the promoter sequence of *Prm2* for voles is highly variable and not alignable from 250 bases upstream of the *Prm2* coding sequence. We therefore chose to limit the *Prm2* promoter alignment to 250 bases for both groups in order to ensure comparability between groups. Our results, therefore, are not directly comparable to those by Martin-Coello et al (2009). *Prm1* and *Prm2* genes are located in close proximity in the genome. A common regulation is therefore possible. Regulatory sequences of *Prm1* and *Prm2* are likely to affect expression of both protamines (Lüke et al 2014b). We present evidence for this co-regulation in voles where we found a negative effect of *Prm1* promoter sequence divergence on both *Prm1* and *Prm2* expression. For mice we found *Prm1* expression to be affected negatively by *Prm1* promoter divergence but positively by *Prm2* promoter divergence. For voles the associations found are quite clear. Higher expression of both protamines seems to be favoured in species with high levels of sperm competition, since changes in *Prm1* promoter sequence seem to decrease protamine expression levels *Prm1* sequence divergence is halted by postcopulatory sexual selection. For mice the relationships seem to be more complex. The analysis of promoter divergence rate is not sufficient to explain regulation of gene expression (Tirosh et al 2008). An extensive comparative analysis of transcription factor binding site divergence and distribution might shed more light on expression regulation of protamines. A more detailed analysis, especially of the highly variable part of the *Prm2* promoter region, which represents the intergenic space between *Prm1* and *Prm2*, will be of interest in future work.

Adaptation of sperm head phenotype through selection on protamine expression

Changes in sperm head dimensions have been found to be important responses to high levels of sperm competition by their effect on sperm swimming velocity (Gómez Montoto et al 2011, Varea Sánchez 2014). Evidence from the present study suggests that sperm head phenotype in rodents is affected at least in part by selective pressures acting on protamine expression levels. These selective pressures seem to differ between groups of species. In mouse species with high levels of sperm competition larger sperm heads in relation to total sperm size are favoured which seems to be at least in part established by an increase in the protamine ratio through selective pressure. In contrast, in voles higher levels of sperm

competition select for a decrease in relative sperm head size seemingly established through a increase in both *Prm1* and *Prm2* expression and a decrease the expression ratio. Since a smaller sperm head size in relation to total sperm length is generally thought to reduce drag (Malo et al 2006, Humphries et al 2008) selecting for larger relative head size in mice seems counterintuitive. However, spermatozoa need various processes to reach the ovum; one of them is the association with the epithelial cell lining the lower isthmus of the oviduct (Suarez 1998, 2008). Attachment to the oviductal wall is crucial for sperm survival (Smith and Yanagimachi 1990). It was proposed that a relative increase in sperm head size might facilitate attachment to the oviductal wall and might therefore increase sperm survival (Gómez Montoto et al 2011). Yet why then is a smaller head relative to total sperm size favoured in voles? It was previously shown in cricetid rodents, including voles and hamsters, that changes in protamine coding sequences result in an increase in sperm head size. Changes in protamine coding sequences are halted by sexual selection in this group of species. It was proposed that a smaller sperm head is beneficial in cricetid rodents by reducing drag and allowing for an increase in sperm swimming velocity (Humphries et al 2008, Lüke et al 2014a). It can be hypothesized that this difference between voles and mice might represent adaptations to a trade-off between sperm survival and sperm swimming velocity affected by differences in parameters of their respective female tract environments.

Consequently, selective pressure due to sperm competition leads to differences in protamine usage between mice and voles resulting in an adaptation of sperm head size. A relative reduction of *Prm2* expression, and therefore an increase in protamine ratio, seems to be the main target of sexual selection in mice. While the protamine ratio does have an effect on sperm head phenotype in voles, an increase of the expression levels of both protamines seems to be the main target of sexual selection.

Importance of protamines in male fertility

Many studies have shown that correct protamine function is critical for male fertility. Additionally, protamines are involved in the nuclear decondensation process following fertilization, which plays an important role in embryonic survival (Perreault et al 1988, Aoki and Carrell 2003, Cho et al 2003, McLay and Clarke 2003) . Even though the protamine ratios differ across mammals (from 0 to 77% PRM2) (Corzett et al 2002), alterations in the species-specific ratios have major effects on male fertility (Cho et al 2001, Haueter et al 2010). Reduced sperm count, motility, and abnormal head morphology have been reported

in human males with altered protamine ratios (de Yebra et al 1998, Carrell and Liu 2001, Aoki et al 2005). Understanding how and why different protamine ratios have evolved through selective pressures not only allows us to understand how evolutionary processes work in general but why the slightest imbalance in protamine expression can lead to male infertility. Here, for example, we found that slight changes in protamine ratio can affect relative sperm head size. Additionally we uncovered evidence for a possible common gene expression regulation for *Prm1* and *Prm2*. To understand why these slight changes are of such importance further studies are needed. These might include 3-dimensional analysis of sperm hydrodynamics, long term experimental evolution and an ample comparison of different taxa and their female tract environments and sperm cell movement patterns. Understanding the impact protamines have on formation and function of the sperm head and their role in male fertility will be crucial to our understanding of reproductive biology.

Lüke L, Varea-Sánchez M, Tourmente M, Roldan ERS (0000) Protamine expression and sperm head phenotype: A comparative analysis**.** *Manuscript in preparation.*

CHAPTER 9
GENERAL DISCUSSION

We were able to identify a complex pattern of selective constraints and sexual selection on protamines as well as evidence for the effect of protamines on sperm head shape and size. The connection sperm competition – protamines – sperm head phenotype, which was examined at various levels, was clearly supported by our findings (Fig. 9.1). Importantly, we found a much more complex pattern of relationships between these three levels than anticipated. Here, a general discussion and interpretion of our findings is presented in the light of previous work. In addition potential lines of future research are identified.

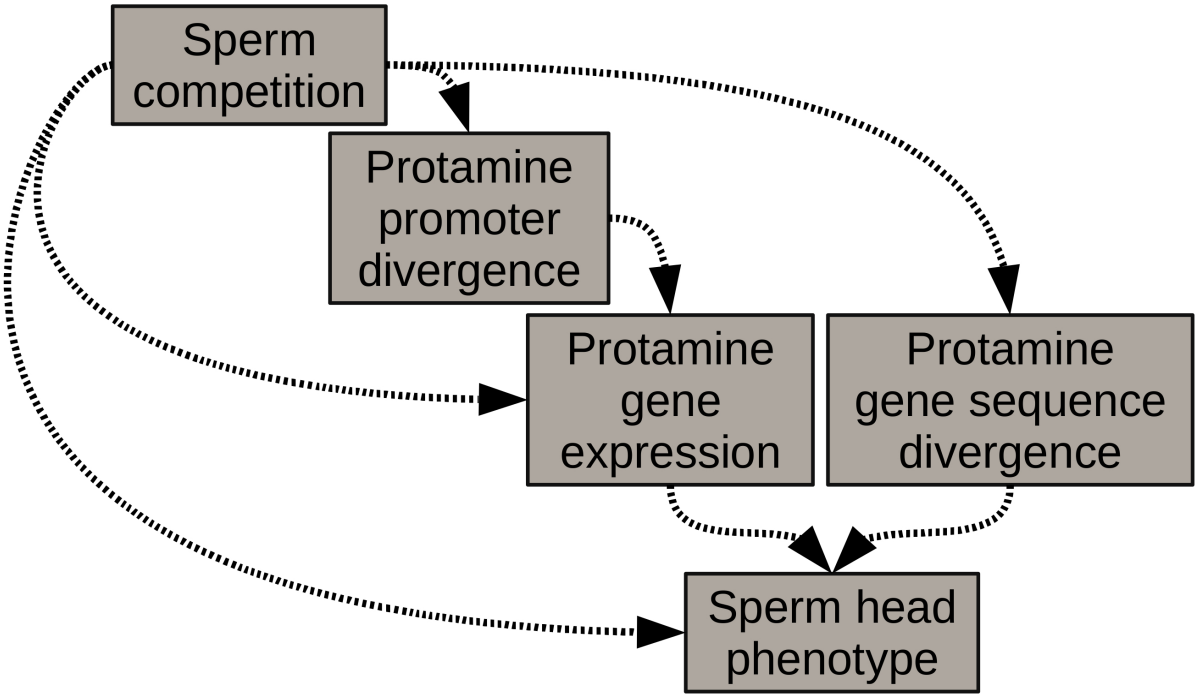


Figure 9.1. Associations between sperm competition, protamines and sperm head phenotype found in this study. Dashed lines represent associations that differed between taxonomic groups analysed or found in only a subset of clades.

PROTAMINE GENE SEQUENCES: SEXUAL SELECTION AND EFFECT ON

Protamines are found in the sperm of protostomes as well as in deuterostomes (Oliva and Dixon 1991). The most commonly accepted scenario for the appearance of protamines is the evolution from a sperm-specific histone H1 through a shift from a lysine-rich histone H1 to the arginine-rich protamine in chordates (Lewis et al 2004).

Protamine 1 is expressed throughout mammals and, according to our findings, seems to be conserved with signs of directed positive selection in most mammalian clades. In primates and cetaceans, however, protamine 1 seems to be under relaxed constraint (Fig. 9.2). Earlier studies hinted a complex pattern of evolution of protamine 1. It was shown that protamine 1 gene sequence across mammals is highly variable, and that it also has highly conserved regions (Oliva and Dixon 1991, Rooney et al 2000, Wyckoff et al 2000). In primates positive selection has been reported (Wyckoff et al 2000), while within rodents conservation of the gene sequence with directed positive selection was previously observed (Lüke et al 2011).

In addition to their evolutionary rates, and selective pressures on protamine gene sequences, we compared ***arginine content*** of protamine 1 between species in order to assess signs of selective pressures acting on protamine 1 composition. The arginine-rich composition of protamines most likely arose through selective pressures posed by internal fertilization (Kansinsky et al 2011). Arginine, as opposed to lysine, provides a higher affinity for DNA binding as well as a greater binding flexibilty due to its guanidinium group (Ausio et al 1984, Cheng et al 2003). According to our results, arginine content of protamine 1 seems to be conserved by sperm competition throughout mammals but especially in rodents and diprotodont marsupials. In these two clades an effect of sexual selection towards higher sequence conservation can be found as well. Additionally, we were able to demonstrate that an increased evolutionary rate seems to result in a loss of arginine while lower arginine content associates with wider sperm heads throughout mammals. In cricetid rodents an association of changes in protamine 1 coding sequence and sperm head size and elongation was evident. Arginine residues within anchoring domains neutralize the charge of the DNA backbone very efficiently and seem to play a role in the activation of egg casein kinase II after fertilization (Ohtsuki et al 1996). Thus, increase in arginine content might result not only in a more condensed and stable sperm chromatin but pose an advantage during post-fertilization stages as well. The maintenance of higher arginine content in rodents and diprotodonts therefore seems to be an adaptation to high sperm

competition levels ensuring efficient chromatin condensation and slim sperm heads.

Eutherian protamine 1 differs from its marsupial counterpart in several important aspects. Eutherian protamine 1 contains 5 to 9 ***cysteine residues***, which allow protamines to form strong disulphide bridges within and between protamines (Balhorn 1982, Oliva and Dixon 1991, Balhorn et al 1995). On the other hand, metatherians, with the exception of the *Planigale* genus (Retief et al 1995a), lack such cysteine residues. Through the formation of disulphide bridges chromatin structure becomes more stable and takes longer to decondense (Cummins 1980).

Marsupial protamine 1 ***sequence was found to be significantly longer*** than eutherian's and positively associated to sperm competition levels. Since in fishes and birds the size of protamines was found to be an important factor in chromatin condensation (Oliva et al 1987, Oliva and Dixon 1991) it can be postulated that marsupials maintain a longer protamine sequence to compensate for the lack of stability resulting from missing disulphide bridges. Sequence length would thus be a key factor in stabilizing chromatin in marsupial mammals.

The selective pressure to increase chromatin stability and rigidity through an appearance of cysteines, increase in arginines, or sequence length, and the resultant effect of sperm head shape, might present an adaptation to key features of sperm function such as sperm swimming speed but also penetration of the ovum. As proposed previously (Bedford and Calvin 1974, Bedford 2008) penetration of the zona pellucida might be facilitated by a more rigid and pointed sperm head. This could be accomplished by a more condensed, stronger chromatin structure and a slimmer sperm head shape. The thickness and composistion of the zona pellucida varies between species (Wassarman 1999) and these might be related to the complexity of adaptations that can be observed in sperm head phenotype and its underlying genotype.

Protamine 2 consists of two functional domains, one of which (mature PRM2) is thought to be a duplication of *Prm*1 and, functionally, it seems to be essentially redundant to *Prm*1. The other functional domain (cleaved PRM2) might be of retroviral origin and of largely unknown function (Krawetz and Dixon 1988, Lüke et al 2011). PRM2 is mainly found in primates and rodents (Balhorn 2007). It undergoes proteolytic cleavage in various successive steps until only the mature PRM2 domain remains bound to the condensed sperm chormatin (Lee et al 1995). While cleaved *Prm*2 is conserved in both clades, mature *Prm*2 is relaxed in rodents and under positive selection in primates (Fig. 9.2). The ***arginine content*** of mature PRM2 is mostly invariable between rodents and primates with

no signs of sexual selection.

In rodents, a higher evolutionary rate of **cleaved *Prm*2** was found to be associated to wider and less elongated sperm heads. Given its general conservation and the association between changes in its coding sequence and sperm head width and elongation, cleaved PRM2 seems to play an important role in sperm head shaping. During the last stages of spermiogenesis the protamine 2 precursor is cleaved over a period of several days while already bound to DNA and when chromatin condensation is taking place (Carre-Eusebe et al 1991, Chauvière et al 1992, Lee et al 1995). Cleaved PRM2 might therefore play a more important role during the actual process of chromatin condensation while mature PRM2 may be involved mainly in the maintenance of the condensed state.

Mature *Prm*2 exhibits less constraint than either cleaved *Prm*2 or *Prm*1. Since it is regarded as functionally redundant to *Prm*1 we propose that mature *Prm*2 is free to change adaptively or to evolve under relaxed constraint due to the existence of a more conserved, functional copy. However, even though protamine function during chromatin condensation is well described and PRM1 and mature PRM2 show strong similarities in amino acid sequence (see Balhorn et al 2007) the possibilty of functional differences between PRM1 and PRM2 have to be further explored in light of the differences in selective pressures we found. It has been proposed that sperm PRM2 content might affect the rate of chromatin decondensation after fertilization (Perreault et al 1988). The rate of chromatin decondensation is an important factor in the post-fertilization process and varies among species. The increased evolutionary rate found for mature *Prm*2 as opposed to *Prm*1 might represent an adaptation to this function. Further studies are needed to clarify whether this is indeed the case.

The ***plasticity of evolutionary and selective patterns*** we found in protamine gene sequences might be explained by adaptations to trade-offs between sperm head morphology, female tract environment, flagellar beating patterns and the influence of other mechanisms of cryptic female choice. For example, arginine loss might result in wider sperm heads. If we assume that this is a disadvantage in rodents and diprotodonts due to the specific way sperm have to move through the female tract, sexual selection would halt the loss of arginine within rodents and diprotodonts. While in other species the loss of arginine also results in wider sperm heads, here, the impact of the female tract of the sperm flagellar beating pattern might result in wider heads being beneficial. The result would be an effect of sexual selection promoting the loss of arginine (without leading to loss of function). When analyzing the influence of sexual selection we therefore need to

take into account that adaptations might be variable and therefore hard to detect when encompassing a wide range of taxonomic groups. Sperm cells need to adapt to their specific fertilization environment (Franzén 1956, Pitnick et al 2009). Given the vast diversity in sperm morphology these environments should be diverse too and pose ample pressure for specific adaptations (Pitnick et al 2009a). Especially in internally fertilizing species those pressures are expected to be highly complex (Pitnick et al 2009b). Therefore, it can be proposed that sperm head size and hook shape are adaptations to diverse sperm functions such as motility, sperm-female tract interactions or sperm-egg interactions (Roldan et al 1991, 1992, Gómez Montoto et al 2011). Additionally, not only adaptations to reach the fertilization site have to be taken into account when trying to understand sperm head phenotype diversity and its underlying genotype. Post-fertilization processes undergone by sperm or sperm-derived structures are poorly known but are thought to be highly complex. Protamines are not only involved in sperm head shaping but have been described to affect chromatin decondensation and protein activation after fertilization takes place (Ohtsuki et al 1996, Brewer et al 1999). These factors, together with their role in sperm head differentiation, sperm function as well as protection of paternal DNA, might have led to this adaptive pattern in protamines balancing conservation of function and adaptations to taxon-specific factors. These adaptations seem to be driven at least in part by postcopulatory sexual selection in the form of sperm competition. Given our results for protamine 1 we suggest that, even though not detected in our analysis, postcopulatory sexual selection may play a role in evolutionary patterns of protamine 2 gene sequences. The identification of this effect, however, could be confounded by the variability between groups of species and the adaptations to the above-mentioned trade-offs.

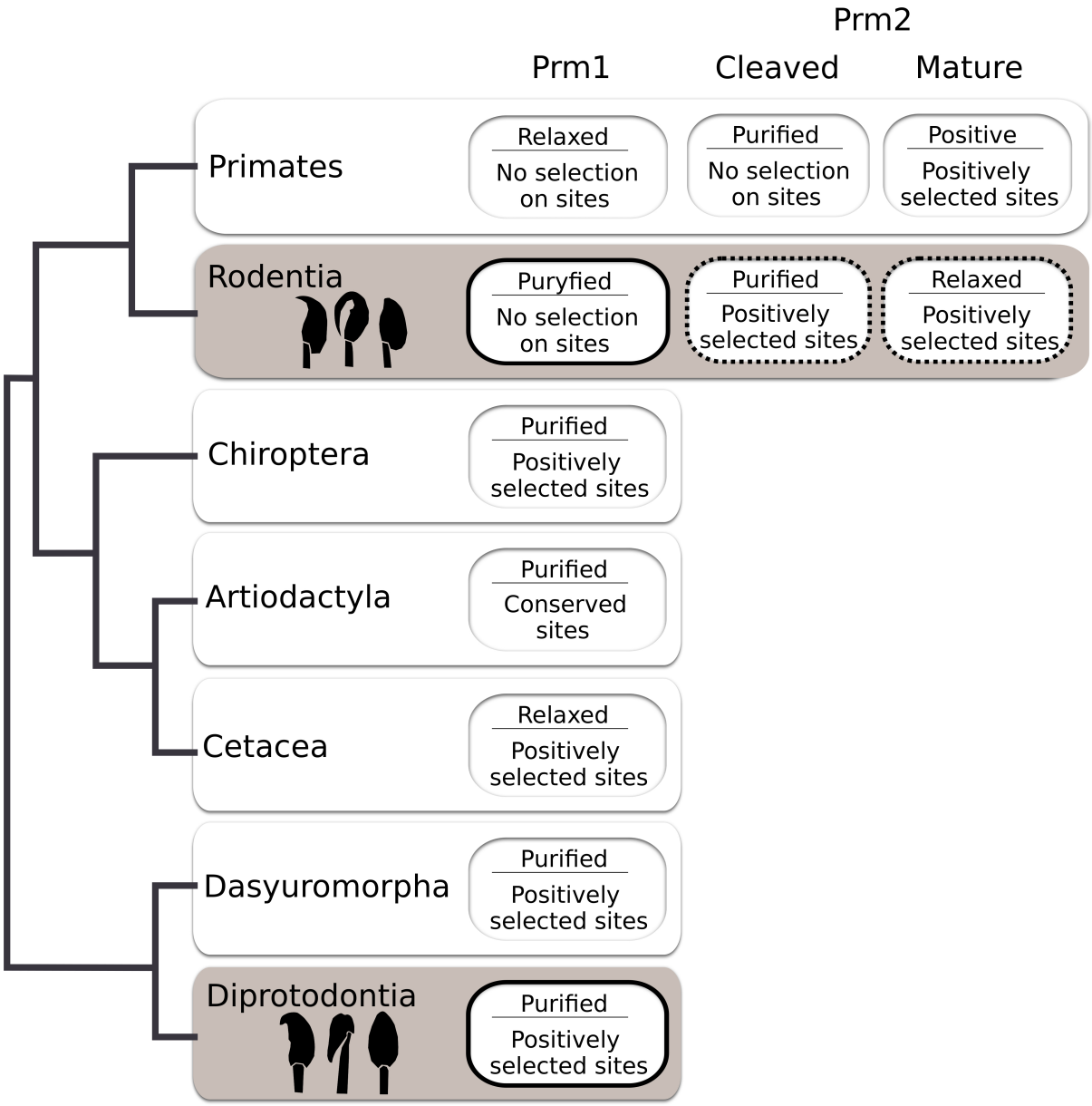


Figure 9.2. Summary of results for selective pressures acting on protamine gene sequences in mammalian clades. Black borders represent evidence for gene sequence evolutionary rate to be affected by sexual selection. Dashed borders represent evidence for gene sequence evolutionary rate to be affected by sexual selection in previous studies including fewer species (Lüke et al 2011).

PROTAMINE GENE EXPRESSION: SEXUAL SELECTION AND EFFECT ON SPERM HEAD PHENOTYPE

When studying the effects of postcopulatory sexual selection changes other than alterations in gene sequences have to be taken into account. Protamine function, and consequently sperm head shape and performance, can also be affected by alterations in regulatory sequences and expression levels alike. Thus, divergence in the promoter region of *Prm*2 has been found to be positively correlated with sperm competition levels and with sperm swimming speed in mice (Martin-Coello et al 2009). With the aim of testing the effects of postcopulatory sexual selection (sperm competition) on gene expression levels we focused on the group of mouse species previously shown to experience the effect of sexual selection on *Prm*2 promoter divergence. We found that relative *Prm*2 expression is reduced in species with higher levels of sperm competition. However, no signal of sexual selection was found on absolute expression of protamine genes. The protamine ratio was found to be associated to changes in sperm head shape in these species.

Protamine expression levels and especially the protamine ratio are important factors in male fertility as demonstrated in several studies in humans and mouse models (de Yebra et al 1998, Carrell and Liu 2001, Oliva 2006). Even though the protamine ratio varies greatly between species (0-77%), the protamine proportion within species is fairly constant (Corzett et al 2002). Modifications in the protamine ratio can result in subfertility and increased DNA fragmentation in humans (Cho et al 2001, Aoki et al. 2005, Oliva 2006). Incomplete PRM2 processing and haplo-insufficieny of PRM2 lead to major alterations in sperm function (de Yebra et al 1998, Cho et al 2003, Torregrosa et al 2006). DNA fragmentation due to aberrant protein ratios might be the result of incorrect DNA packaging leading to an insufficient protection of the paternal DNA. Consistent with these findings, our results suggest the protamine ratio to be of great importance for sperm competitiveness and sperm head phenotype.

Earlier studies found that species lacking PRM2 or producing very little PRM2 relative to PRM1 decondense DNA more slowly after fertilization (Perreault et al 1988, Corzett et al 2002). A higher compaction of chromatin might be the reason for slower decondensation. A relative reduction in PRM2 might therefore alter the hydrodynamic capacities of the sperm head resulting in a more advantageous head shape in the studied group of species. Reduction in the relative abundance of PRM2 therefore seems to enhance sperm

competitive ability in mice and affect sperm head shape. A comparative study of DNA fragmentation and chromatin decondensation rates, in relation to differences in species-specific protamine ratios, would thus be important to further understand relations between protamine function, sperm competitiveness and male fertility.

PROTAMINES: FROM GENOTYPE TO PHENOTYPE

Given the complex patterns of postcopulatory sexual selection found between groups of species, and the high diversity of head shapes and sperm competition in rodents, we performed a genotype-phenotype association study. To this end we compared two rodent taxa (mice and voles) to further investigate differences in adaptation mechanisms of protamines to sperm competition and to identify associations between protamine regulation, protamine expression and sperm head phenotype. Mice and voles were regarded as adequate models because they have a very similar range in sperm competition levels but vary greatly in sperm head morphology (Varea Sánchez 2014). Additionally, these two groups show different divergence times (mice: 5 MYA; voles: 18 MYA, Steppan et al 2004) which allows for a comparison of adaptations early stages, during diversification, versus later stages after the initial diversification.

Voles were found to express more *Prm*1 mRNA than mice while the two groups do not differ in their *Prm*2 expression. This results in a higher protamine ratio in voles than in mice. Divergence of the *Prm*1 promoter is significantly higher in voles than in mice while the divergence of the *Prm*2 promoter does not differ significantly between groups. We tested if these differences in protamine expression are driven by postcopulatory sexual selection and if they could lead to the marked differences found in sperm head phenotype between these species (Varea Sánchez 2014). In voles, protamine ratios and expression levels seem to be driven by sexual selection. Thus, within voles, species with high levels of sperm competition show lower ratios and but higher total expression levels. In contrast, within mice an increase in protamine ratio by a relative reduction of protamine 2 expression is favoured by sperm competition.

Changes in sperm head morphology are important responses to high levels of sperm competition, perhaps due to their effect on sperm swimming velocity (Gómez Montoto et al 2011, Varea Sánchez 2014). In voles, the selective pressure affecting protamine expression was found to be associated to a smaller relative sperm head size. Thus, in

voles, a smaller head relative to total sperm size seems to be favoured in species with higher levels of sperm competition. As we showed for cricetid rodents (voles and hamsters) changes in protamine coding sequences result in an increase in sperm head size; therefore evolutionary rate is halted in protamine 2 in this group of species (Lüke et al 2011). We propose that a smaller sperm head would allow for an increase in sperm swimming velocity because of the resulting reduction in drag (Humphries et al 2008) and therefore would be advantageous in cricetid rodents.

In contrast, in mice, selective pressures affecting protamine ratios seem to result in an increase in relative sperm head size. This seems to disagree with the idea that a decrease in drag would be followed by an increase in sperm velocity as proposed for voles. However, spermatozoa are involved in multiple processes until they reach the fertilization site. During their transit along the female tract sperm cells need to associate with the epithelium of the oviduct and remain attached which increases their life span (reviewed in Suarez 2008). It was proposed that a relative increase in sperm head size facilitate sperm attachment to the oviduct and thus benefits sperm survival (Gómez Montoto et al 2011). It is conceivable that the contradiction we found between vole and mouse protamines and head size selection represents adaptations to a trade-off between sperm survival and sperm swimming velocity.

Protamines are affected by postcopulatory sexual selection in both voles and mice but this selection drives contrasting expression patterns resulting in different head phenotypes. We propose that in voles and mice sperm competitiveness is improved by adaptation of relative protamine expression leading to more beneficial head size. The type of head modifications favoured in each group of species seem to be influenced by many factors, possibly including female tract environments, flagellar beating patterns, and cryptic female choice. These results open up opportunities for future work in order to understand the complex mechanisms shaping sperm head phenotype and protamine evolutionary patterns.

GENERAL OUTCOME

Protamines, due to their important role in sperm formation and male fertility, as well as their unusual evolutionary and selection patterns, have been shown here to be an excellent model to study processes of sexual selection and genotype-phenotype associations. We were able to present evidence for the effect of sperm competition on protamine sequence evolution and expression. Likewise we were able to draw a connection between protamine sequence divergence, expression levels and sperm head phenotype (Fig. 9.1).

Our understanding of why the patterns of selective constraints and sexual selection we found are so plastic is far from complete. In order to improve our understanding of this complex network (sperm competition – protamines – sperm head phenotype) further studies are needed. In addition, the functionality of protamines does not end with chromatin compaction and sperm head shaping. Furture studies should also focus on additional funtional properties of protamines, and incorporate our findings, to elucidate the complex patterns of protamine evolution.

FUTURE DIRECTIONS

We studied protamine gene expression, its selection and effect on sperm head phenotype. Our results, and their potential implications, open up new roads for research, with several additional objectives to be addressed in the future.

1. mRNA expression levels do not always correlate with the amount of protein found after translation and might change again after posttranslational modification (Greenbaum et al 2003). A comparative study of protamine gene expression, protein expression levels during spermiogenesis, and protein expression levels in mature sperm can lead to more detailed and robust conclusions as to how sperm competition affects protamine expression and how sperm head phenotype is modified.

2. The role of cleaved PRM2 is still unclear and differences in mature PRM2 and PRM1 still have to be examined in detail (Mateo et al 2011, Lüke et al 2014a, 2015b). Differences in timing and localization of PRM1 and PRM2 inside the nucleus during chromatin

condensation may exist between species. These processes may be affected by sperm competition and, thus, show different patterns between species exhibiting differences in their sperm competition levels. A comparative study of immunohistological and immunocytochemical detection of protamines in testes sections, as well as in mature sperm cells, would allow for a detailed analysis of spatial distribution and timing of expression during spermiogenesis and in mature functional sperm cells.

3. The main role of protamines is to ensure a tightly packed, protected sperm chromatin and correct decondensation after fertilization. Both of these processes are affected by aberrant protamine expression and play a very important role in male fertility (Aoki and Carrell 2003, Cho et al 2003, Torregrosa et al 2006). Differences between species, however, can be found in protamine expression rates and they may relate to differences in normal chromatin compaction and decondensation rates. A comparative study evaluating degree of compaction and the rate of chromatin decondensation in species with differences in protamine expression patterns and sperm competition levels would let us draw more detailed conclusions of protamine function and selection.

4. The relationship between protamine regulatory sequences and protamine expression is far from clear (Lüke et al 2014b, 2015c). We were able to find an association between promoter divergence rates and expression. However, in order to understand how protamines are regulated more detailed analyses are necessary. A comparison of transcription factor binding site distribution and divergence rates of the promoter regions would be required. Additionally, a detailed analysis of the highly variable protamine intergenic region (Lüke et al 2015c), including a comparison of length, transcription factor binding site, and transposable element distribution might render important insights into processes of regulatory evolution and regulation of expression in general.

CHAPTER 10

CONCLUSIONES

1. La selección sexual afecta a las protaminas en distinto grado en mamíferos. Los patrones evolutivos son diversos, y unas adaptaciones complejas se ven promovidas por una diversidad de presiones selectivas entre diferentes grupos de especies.

2. La protamina 1 y la protamina 2, así como los dos dominios de la protamina 2, se ven afectadas en forma diferente por las restricciones selectivas y la selección sexual postcópula.

3. El dominio de la protamina 2 que se cliva y elimina ("protamina 2 clivada") está conservada con selección direccional positiva. La protamina 1 está conservada principalmente por selección direccional positiva. El dominio de protamina 2 que permanece unido al ADN ("protamina 2 madura") está bajo restricción baja o está libre para evolucionar en forma adaptativa debido a la redundancia funcional con la protamina 1.

4. En los mamíferos, la protamina 1 parece evolucionar bajo selección purificante, con selección direccional en algunos sitios específicos de codones, con excepción de los primates y cetáceos en los cuales se ha encontrado restricción relajada. Se identificó en mamíferos una tendencia generalizada de la selección sexual sobre el mantenimiento de altos contenidos de arginina en protamina 1, que resultan en cabezas espermáticas más angostas.

5. Los cambios en las secuencias codificantes de la protamina 1 y la protamina 2 clivada afectan al tamaño de la cabeza espermática en mamíferos, mientras que los ratios de expresión de protaminas parecen afectar la forma y el tamaño de la cabeza espermática al menos en roedores.

6. La selección sexual promueve la adaptación diferencial de los ratios de expresión de protaminas en dos subfamilias de roedores, resultando en diferencias esenciales en la morfología de la cabeza espermática de estos dos grupos.

7. Hay una ausencia de residuos de cisteína en la secuencia de protamina 1 de los marsupiales, lo que resulta en una incapacidad de estabilizar la cromatina a través de puentes disulfuro entre protaminas. En este taxón, las variaciones en la longitud de la secuencia de protamina 1 están positivamente relacionadas con los niveles de competición espermática.

CONCLUSIONS

1. Sexual selection affects protamines to varying degrees throughout mammals. Evoutionary patterns are diverse, and complex adaptations are driven by a diversity of selective pressures between groups of species.

2. Protamine 1 and protamine 2, as well as the two domains of protamine 2, are differently affected by selective constraints and postcopulatory sexual selection.

3. The protamine 2 domain that is cleaved off ("cleaved protamine 2") is conserved with directed positive selection. Protamine 1 is mainly conserved with directed positive selection. The protamine 2 domain that remains bound to DNA ("mature protamine 2") is under lower constraint or free to evolve adaptively due to functional redundancy to protamine 1.

4. Troughout mammals protamine 1 seems to evolve under purifying selection with directed selection on specific codon sites, with the exception of primates and cetaceans in which a relaxed constraint is found. A common trend across mammals is for sexual selection to maintain high arginine content in protamine 1 resulting in slimmer sperm heads.

5. Changes in protamine 1 and cleaved protamine 2 coding sequences affect sperm head size in mammals, while protamine expression ratios seem to affect sperm head size and shape at least in rodents.

6. Sexual selection drives differential adaptation of protamine expression ratios in two rodent subfamilies resulting in essential differences in sperm head morphology between these two groups.

7. There is a lack of cysteine residues in the protamine 1 sequence of marsupials, which results in an inability to stabilize chromatin through disulphide bridges between protamines. In this taxon, variations in protamine 1 sequence length are positively related to levels of sperm competition.

CHAPTER 11

REFERENCES

Abzhanov A, Protas M, Grant R, Grant PR, Tabin CJ (2004) Bmp4 and morphological variation of beaks in Darwin's finches. Science 305:1462-1465.

Abzhanov A, Winston PK, Hartmann C, Grant R, Grant PR, Tabin CJ (2006) The calmodulin pathway and evolution of elongated beak morphology in Darwin's finches. Nature 442:563-567.

Aoki VW, Carrell DT (2003) Human protamines and the developing spermatid: their structure, function, expression and relationship with male infertility. Asian J Androl 5:315-324.

Aoki VW, Christensen GL, Atkins JF, Carrell DT (2006) Identification of novel polymorphisms in the nuclear protein genes and their relationship with human sperm protamine deficiency and severe male infertility. Fertil Steril 86:1416-1422.

Aoki VW, Liu L, Carrell DT (2005) Identification and evaluation of a novel sperm protamine abnormality in a population of infertile males. Hum Reprod 20:1298-1306.

Ausio J, Greulich KO, Haas, E, Wachtel E (1984) Characterization of the fluorescence of the protamine thynnine and studies of binding to double-stranded DNA. Biopolymers 23:2559–2571.

Balhorn MC, Balhorn R (2011) Protamines: Structure and Function (Editorial). Proteine Peptide Lett 8:753.

Balhorn R (1982) A model for the structure of chromatin in mammalian sperm. J Cell Biol 93:298–305.

Balhorn R (1989) Mammalian proteins, structure and molecular interaction. In: Adolph KW, editor. Molecular Biology of Chromosome Function. New York: Springer Verlag. pp. 366-395.

Balhorn R (2007) The protamine family of sperm nuclear proteins. Genome Biol 8:227.

Balhorn R, Corzett M, Mazrimas JA (1992) Formation of intraprotamine disulfides in vitro. Archives of Biochemistry and Biophysics 296:384-393

Balhorn R, Cosman M, Thornton K, Krishnan VV, Corzett M, Bench G, Kramer C, Lee J IV, Hud NV, Allen M, et al (1999) Protamine Mediated Conden- sation of DNA in Mammalian Sperm, in The Male Gamete: From Basic Knowledge to Clinical Applica- tions, C. Gagnon, Editor. Cache River Press: Vienna, IL. 55–70.

Balhorn R, Hud NV, Corzett M, Mazrimas J (1995) Importance of protamine phosphorylation to histone displacement in spermatids:can the disruption of this process be used for male contraception. Livermore California: Lawrence Livermore National Laboratory. UCRL-ID-120383.

Balhorn R, Reed S, Tanphaichitr N (1988) Aberrant protamine 1/protamine 2 ratios in sperm of infertile human males. Experientia 44:52–55.

Bedford JM (2008) Puzzles of mammalian fertilization – and beyond. Int J Dev Biol 52:415-426

Bedford JM, Calvin HI (1974) The occurrence and possible functional significance of -S-S- crosslinks in sperm heads, with particular reference to eutherian mammals. J Exp Zool 188:137-55.

Belokopytova IA, Kostyleva EI, Tomilin AN, Vorob'ev VI (1993) Human male infertility may be due to a decrease of the protamine P2 content in sperm. Mol Reprod Dev 34:53-57.

Birkhead TR, Hosken DJ, Pitnick S (2009) Sperm Biology - An Evolutionary Perspective. Oxford: Academic Press. 674 p.

Birkhead TR, Martinez JG, Burke T, Froman DP (1999) Sperm mobility determines the outcome of sperm competition in the domestic fowl. Proc Roy Soc Lond Ser B 266:1759–1764.

Birkhead TR, Møller AP (1998) Sperm Competition and Sexual Selection. London: Academic Press. 826 p.

Breed WG, Taylor J (2000) Body mass, testes mass, and sperm size in murine rodents. J Mammal 81:758-768

Brewer L, Corzett M, Balhorn R (2002) Condensation of DNA by spermatid basic nuclear proteins. J Biol Chem 277:38895-900.

Brewer LR (1999) Protamine-induced condensation and decondensation of the same DNA molecule. Science 286:120-123.

Briskie JV, Montgomerie R, Birkhead TR (1997) The evolution of sperm size in birds. Evolution, 51:937–945.

Campbell NA, Atchley WR (1981) The geometry of canonical variate analysis. Syst Zool 30, 268-280.

Carleton KL, Kocher TD. (2001) Cone opsin genes of African cichlid fishes: tuning spectral sensitivity by differential gene expression. Mol Biol Evol 18:1540-1550.

Carre-Eusebe D, Lederer F, Le KH, Elsevier SM (1991) Processing of the precursor of protamine P2 in mouse. Peptide mapping and N-terminal sequence analysis of intermediates. Biochem J 277: 39.

Carrell DT, Emery BR, Hammoud S (2007) Altered protamine expression and diminished spermatogenesis:what is the link? Hum Reprod Update 13:313–327.

Carrell DT, Emery BR, Liu L (1999) Characterization of aneuploidy rates, protamine levels, ultrastructure, and functional ability of round-headed sperm from two siblings and implications for intracytoplasmic sperm injection. Fertil Steril 71:511-516.

Carrell DT, Liu L (2001) Altered protamine 2 expression is uncommon in donors of known fertility, but common among men with poorfertilizing capacity, and may reflect other abnormalities of spermiogenesis. J Androl 22:604-610.

Carroll SB (2005) Evolution at two levels: On genes and form. PLoS Biol 3:1159-1166.

Chauvière M, Martinage A, Debarle M, Sautière P, Chevaillier P (1992) Molecular characterization of six intermediate proteins in the processing of mouse protamine P2 precursor. Eur J Biochem 204:759-65.

Cheng AC, Chen WW, Fuhrmann CN, Frankel AD (2003) Recognition of nucleic acid bases and base-pairs by hydrogen bonding to amino acid side-chains. J Mol Biol 327:781–796.

Cho C, Jung-Ha H, Willis WD, Goulding EH, Stein P, Xu Z, Schultz RM, Hecht NB, Eddy EM (2003) Protamine-2 deficiency leads to sperm DNA damage and embryo death in mice. Biol Reprod 69:211-217.

Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi YC, Hecht NB, Eddy EM (2001) Haploinsufficiency of protamine-1 or -2 causes infertility in mice. Nat Genet 28:82–86.

Clark AG, Civetta A (2000) Evolutionary biology: protamine wars. Nature 403:261-263.

Cohen J (1977) Reproduction. London, UK: Butterworths.

Corzett M, Kramer C, Blacher R, Mazrimas J, Balhorn R (1999) Analysis of hamster protamines, primary sequence and species distribution. Mol Reprod Dev 282:273-282.

Corzett M, Mazrimas J, Balhorn R (2002) Protamine 1, protamine 2 stoichiometry in the sperm of eutherian mammals. Mol Reprod Dev 61:519-27.

Cree LH, Balhorn R, Brewer LR (2011) Single molecule studies of DNA-protamine interactions. Protein Pept Lett 18:802–810.

Cummins JM (1980) Decondensation of sperm

nuclei of Australian marsupials:Effects of air drying and of calcium and magnesium. Gamete Res 3:351-367.

Cummins JM, Woodall PF (1985) On mammalian sperm dimensions. J Reprod Fertil 75:153–175.

Cummins JM, Yanagimachi R (1982) Sperm-egg ratios and the site of the acrosome reaction during in vivo fertilization in the hamster. Gamete Res 5:239-256.

Dadoune JP (2003) Expression of mammalian spermatozoal nucleoproteins. Microscopy Research and Technique 61:56–75.

Darwin C (1871) The Descent of Man, and Selection in Relation to Sex. London: John Murray

de Mateo S, Ramos L, de Boer P, Meistrich M, Oliva R (2011) Protamine 2 precursors and Processing. Protein Peptide Lett 18:000-000.

de Yebra L, Ballescá JL, Vanrell JA, Corzett M, Balhorn R, Oliva R (1998) Detection of P2 precursors in the sperm cells of infertile patients who have reduced protamine P2 levels. Fertil Steril 69:755-9.

Dorus S, Evans PD, Wyckoff GJ, Choi SS, Lahn BT (2004) Rate of molecular evolution of the seminal protein gene SEMG2 correlates with levels of female promiscuity. Nat Genet 36:1326-1329.

Dorus S, Karr TL (2009) Sperm proteomics and genomics. In TR Birkhead, DJ Hosken, S Pitnick (Eds.), Sperm Biology: An Evolutionary Perspective (1st ed.). Elsevier.

Fabre PH, Hautier L, Dimitrov D, Douzery EJP (2012) A glimpse on the pattern of rodent diversification: a phylogenetic approach. BMC Evol Biol 12:88.

Felsenstein J (1985) Phylogenies and the comparative method. Am Nat 125:1-15.

Findlay GD, Swanson WJ (2010) Proteomics enhances evolutionary and functional analysis of reproductive proteins. BioEssays 32:26-36.

Finn S, Civetta A (2010) Sexual selection and the molecular evolution of ADAM proteins. J Mol Evol 71:231-240.

Firman RC, Simmons LW (2009) Experimental evolution of sperm quality via postcopulatory sexual selection in house mice. Evolution 64:1245-1256.

Fitzpatrick JL, Montgomerie R, Desjardins JK, Stiver K, Kolm N, Balshine S (2009) Female promiscuity promotes the evolution of faster sperm in cichlid fishes. PNAS 106:1128–32.

Florman HM, Ducibella T (2006) Fertilization in mammals. In Knobil and Neill's Physiology of Reproduction, pp 55–112. Ed. JD Neill. San Diego: Elsevier.

Franzén A (1956) On spermiogenesis, morphology of the spermatozoon, and biology of fertilization among invertebrates. Zoologiska Bidrag Fran Uppsala, 31:355–482.

Freckleton R, Harvey PH, Pagel MD (2002) Phylogenetic analysis and comparative data:a test and review of evidence. Am Nat 160:712–726.

Froman DP, Feltmann AJ, Rhoads ML, Kirby JD (1999) Sperm mobility: A primary determinant of fertility in the domestic fowl (Gallus domesticus). Biol Reprod 61:400–405.

Gage MJG, Macfarlane CP, Yeates S, Ward RG, Searle JB, et al (2004) Spermatozoal traits and sperm competition in Atlantic salmon: Relative sperm velocity is the primary determinant of fertilization success. Curr Biol 14:44-47.

Galewski T, Tilak M, Sanchez S, Chevret P, Paradis E, et al (2006) The evolutionary radiation of Arvicolinae rodents (voles and lemmings), relative contribution of nuclear and mitochondrial DNA phylogenies. BMC Evol Biol 6:80.

Gilchrist E, Haughn G (2005) TILLING without A plough: A new method with applications for reverse genetics. Curr Opin Plant Biol 8:211–215.

Goldman N, Yang Z (1994) A codon-based model of nucleotide substitution for protein-coding DNA sequences. Mol Biol Evol 11:725–736.

Gomendio M, Harcourt H, Roldan ERS (1998) Sperm Competition in Mammals. In: Birkhead TR, Møller AP. eds. Sperm Competition and Sexual Selection. London: Academic Press. pp. 667-751.

Gomendio M, Martin-Coello J, Crespo C, Magaña C, Roldan ERS (2006) Sperm competition enhances functional capacity of mammalian spermatozoa. PNAS USA 103:15113-15117.

Gomendio M, Roldan ERS (1991) Sperm competition influences sperm size in mammals. PRSB 243:181–185.

Gomendio M, Roldan ERS (2008) Implications of diversity in sperm size and function for sperm competition and fertility. Int J Dev Biol 52:439–447.

Gómez Montoto L, Magaña C, Tourmente M, Martín-Coello J, Crespo C, et al (2011) Sperm competition, sperm numbers and sperm quality in Muroid rodents. PLoS One 6:e18173.

Gómez Montoto L, Varea Sánchez M, Tourmente M, Martín-Coello J, Luque-Larena JJ, Gomendio M, Roldan ERS (2011) Sperm competition differentially affects swimming velocity and size of spermatozoa from closely related muroid rodents:head first. Reproduction 142:819–830.

Goodall C (1991) Procrustes methods in the statistical analysis of shape. PRSB 53:285-339.

Greenbaum D, Colangelo C, Williams K, Gerstein M (2003) Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol. 4:117.

Guénet JL, Bonhomme F (2003) Wild mice: an ever-increasing contribution to a popular mammalian model. Trends Genet 19:24–31.

Hall T (1999) BioEdit, a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids S 41:95-98.

Harvey PH, Pagel MD (1991) The comparative method in evolutionary Biology. New York:Oxford University Press.

Haueter S, Kawsumi M, Asner I, Brykczynska U, Cinelli P, Moisyadi S, Burki K, Peters AHFM, Pelczar P (2010) Genetic vasectomy-overexpression of Prm1-EGFP fusion protein in elongating spermatids causes dominant male sterility in mice. Genesis 48:151-160.

Hecht NB (1993) Gene expression during male germ cell development. In Cell and molecular Biology of the testis. Edited by Desjardins C, Ewing LL. New York: Oxford University Press:400-432.

Herlyn H, Zischler H (2007) Sequence evolution of the sperm ligand zonadhesin correlates negatively with body weight dimorphism in primates. Evolution 61:289–298.

He XJ, Ruan J, Du WD, Chen G, Zhou Y, Xu S, Zuo XB, Cao YX, Zhang XJ (2012) Prm1 variant rs35576928. Arg > Ser is associated with defective spermatogenesis in the Chinese Han population. Reprod Biomed Online 25:627-634.

Hoekstra HE, Coyne JA. 2007 The locus of evolution: evo devo and the genetics of adaptation. Evolution 61, 995-1016.

Huber, PJ (1981) Robust statistics. New York: John Wiley.

Huerta-Cepas J, Dopazo J, Gabaldon T (2010) ETE, a python environment for tree exploration. BMC Bioinformatics 11:24.

Humphries S, Evans JP, Simmons LW (2008) Sperm competition:linking form to function. BMC Evol Biol 8:319

Hunter FM, Birkhead TR (2002) Sperm viability and sperm competition in insects. Current Biology 12:121–123.

Immler S, Moore HD, Breed WG, Birkhead TR (2007) By hook or by crook? Morphometry, competition and cooperation in rodent sperm. PLoS One 2:e170.

Jaarola M, Martinkova N, Gunduz I, Brunhoff C,

Zima J, et al (2004) Molecular phylogeny of the speciose vole genus Microtus (Arvicolinae, Rodentia) inferred from mitochondrial DNA sequences. Mol Phylogenet Evol 33:647-663.

Jodar M, Oriola J, Mestre G, Castillo J, Giwercman A, Vidal-Taboada JM, Ballescá JL, Oliva R (2010) Polymorphisms, haplotypes and mutations in the protamine 1 and 2 genes. Int J Androl 34:1–16.

Jones FC, Grabherr MG, Chan YF, Russell P, Mauceli E et al (2012) The genomic basis of adaptive evolution in threespine sticklebacks. Nature 484:55-61.

Kasinsky HE, Eirin-Lopez JM, Ausió J (2011) Protamines: structural complexity, evolution and chromatin patterning. Protein Pept Lett 18:755–71.

Kenagy GJ, Trombulak SC (1986) Size of mammalian testes in relation to body size. J Mammal 67:1-22.

Kendall D (1986) The diffusion of shape. Adv Appl Probab 9:428-430.

Kierzenbaum AL, Tres LL (1975) Structural and transcriptional features of the mouse spermatid chromosome. J Cell Biol 65:258-270.

Kingan SB, Tatar M, Rand DM (2003) Reduced polymorphism in the chimpanzee semen coagulating protein, semenogelin I. J Mol Evol 57:159-69.

King MC, Wilson AC (1975) Evolution at two levels in humans and chimpanzees. Science 188:107-188.

Kleene KC, Bagarova J (2008) Comparative genomics reveals gene-specific and shared regulatory sequences in the spermatid-expressed mammalian Odf1, Prm1, Prm2, Tnp1 and Tnp2 genes. Genomics 92:101-106.

Klingenberg CP (2011) MorphoJ: an integrated software package for geometric morphometrics. Mol Ecol Res 11:353-357.

Klingenberg CP, Gidaszewski NA (2010) Testing and quantifiying phylogenetic signals and homoplasy in morphometric data. Syst Biol59:245-261.

Krawetz SA, Dixon GH (1988) Sequence similarities of the protamine genes, implications for regulation and evolution. J Mol Evol 27:291–297.

Lee K, Haugen HS, Clegg CH, Braun RE (1995) Premature translation of protamine 1 mRNA causes precocious nuclear condensation and arrests spermatid differentiation in mice. PNAS USA 92:12451-12455.

Lewis JD, Saperas N, Song Y, Zamora MJ, Chiva M, Ausió J (2004) Histone H1 and the origin of protamines. PNAS 101:41-48.

Lüke L, Campbell P, Sánchez MV, Nachman MW, Roldan ERS (2014b) Sexual selection on protamine and transition nuclear protein expression in mouse species. PRSB 281:20133359.

Lüke L, Tourmente M, Dopazo H, Serra F, Roldan ERS (0000b) Selective constraints on protamine 2 in primates and rodents. submitted.

Lüke L, Tourmente M, Roldan ERS (0000a) Sexual selection of protamine 1 in mammals. submitted.

Lüke L, Varea Sanchez M, Tourmente M, Ortiz D, Roldan ERS (0000c) Protamines and sperm head phenotype: A comparative analysis. in preparation.

Lüke L, Vicens A, Serra F, Luque-Larena JJ, Dopazo H, Roldan ERS, Gomendio M (2011) Sexual selection halts the relaxation of protamine 2 among rodents. PLoS One 6:e29247.

Lüke L, Vicens A, Tourmente M, Roldan ERS (2014a) Evolution of protamine genes and changes in sperm head phenotype in rodents. Biol Reprod 90:67.

Lundrigan BL, Jansa S, Tucker, PK (2002) Phylogenetic relationships in the genus Mus, based on paternally, maternally, and biparentally inherited characters. Syst Biol 51:410-431.

MacLeod CD (2010) The relationship between body mass and relative investment in testes mass in cetaceans:Implications for inferring interspecific variations in the extent of sperm competition. Mar Mammal Sci 26:370–380.

Mali P, Kaipia A, Kangasniemi M, Toppari J, Sandberg M, Hecht NB, Parvinen M (1989) Stage-specific expression of nucleo-protein mRNAs during rat and mouse spermiogenesis. Reprod. Fertil Dev 1:369-382.

Malo AF, Gomendio M, Garde J, Lang-Lenton B, Soler AJ, et al (2006) Sperm design and sperm function. Biol Lett 2:246–249.

Manceau M, Domingues VS, Mallarino R, Hoekstra HE (2011) The developmental role of agouti in color pattern evolution. Science 331:1062-1065.

Martin-Coello J, Dopazo H, Arbiza L, Ausió J, Roldan ERS, Gomendio M (2009) Sexual selection drives weak positive selection in protamine genes and high promoter divergence, enhancing sperm competitiveness. PRSB 276:2427.

Martins EP (2004) COMPARE, version 46b Computer programs for the statistical analysis of comparative data. At http://compare.bio.indiana.edu/. Department of Biology, Indiana University, Bloomington IN.

McLay DW, Clarke HJ (2003) Remodelling the paternal chromatin at fertilization in mammals. Reproduction 125:625-633.

Meistrich ML, Mohapatra B, Shirley CR, Zhao M (2003) Roles of transition nuclear proteins in spermiogenesis. Chromosoma 111:483-488.

Møller AP (1989) Ejaculate quality, testes size and sperm production in mammals. Funct Ecol 3:91-96.

Montgomery SH, Capellini I, Venditti C, Barton RA, Mundy NI (2011) Adaptive evolution of four microcephaly genes and the evolution of brain size in anthropoid primates. Mol Biol Evol 28:625–38.

Montgomery SH, Mundy NI (2012) Adaptive evolution of four microcephaly genes and the evolution of brain size in anthropoid primates. Evolution 66:927-32

Nayernia K, Drabent B, Adham IM, Moschner M, Wolf S, Meinhardt A, Engel W (2003) Mice lacking three germ cell expressed genes are fertile. Biol Reprod 69:1973-1978.

Neumann K, Michaux J, Lebedev V, Yigit N, Colak E, et al (2006) Molecular phylogeny of the Cricetinae subfamily based on the mitochondrial cytochrome b and 12S rRNA genes and the nuclear vWF gene. Mol Phylogenet Evol 39:135-148.

Ohtsuki K, Nishikawa Y, Saito H, Munakata H, Kato T (1996) DNA-binding sperm proteins with oligo-arginine clusters function as potent activators for egg CK-II. FEBS Lett 378:115–120.

Oliva R (2006) Protamines and male infertility. Hum Reprod Update 12:417.

Oliva R, Bazett-Jones D, Mezquita C, Dixon GH (1987) Factors affecting nucleosome disassembly by protamines in vitro:histone hyperacetylation and chromatin structure, time dependence and the size of the sperm nuclear proteins. J Biol Chem 262:17016-17025.

Oliva R, Dixon GH (1991) Vertebrate protamine genes and the histone-to-protamine replacement reaction. Prog Nucleic Acid Res 40:25–94.

Pagel, M (1999) The maximum likelihood approach to reconstructing ancestral character states of discrete characters on phylogenies. Syst Biol 48:612-622.

Parker GA (1970) Sperm competition and its evolutionary consequences in the insects. Biol Rev 45:525-567.

Parker GA, Pizzari T (2010) Sperm competition and ejaculate economics. Biol Rev 85: 897-934.

Perreault SD, Barbee RR, Elstein KH, Zucker RM, Keefer CL (1988) Interspecies differences in the stability of mammalian sperm nuclei assessed in vivo by sperm microinjection and in vitro by flow cytometry. Biol Reprod 39:157-167.

Pitnick S, Hosken DJ, Birkhead TR (2009a) Sperm diversity. In TR Birkhead, DJ Hosken, S Pitnick (Eds.) Sperm Biology: An Evolutionary Perspective (1st ed.). Elsevier.

Pitnick S, Miller GT, Schneider K, Markow TA (2003) Ejaculate–female coevo- lution in Drosophila mojavensis. PRSB 270:1507–1512.

Pitnick S, Wolfner MF, Suarez SS (2009b) Ejaculate– and sperm–female interactions. In TR Birkhead, DJ Hosken, S Pitnick (Eds.) Sperm Biology: An Evolutionary Perspective (1st ed.). Elsevier.

Pointer M a, Kamilar JM, Warmuth V, Chester SGB, Delsuc F, Mundy NI, Asher RJ, Bradley BJ (2012) RUNX2 tandem repeats and the evolution of facial length in placental mammals. BMC Evol Biol 12:103.

Prothmann A, Laube I, Dietz J, Roos C, Mengel K, Zischler H, Herlyn H (2012) Sexual size dimorphism predicts rates of sequence evolution of SPerm Adhesion Molecule 1 (SPAM1, also PH-20) in monkeys, but not in hominoids. apes including humans. Mol Phylogenet Evol 63:52–63.

Queralt R, Adroer R, Oliva R, Winkfein RJ, Retief JD, Dixon GH (1995) Evolution of protamine P1 genes in mammals. J Mol Evol 40:601-607.

Queralt R, Oliva R (1993) Identification of conserved potential regulatory sequences of the protamine-encoding P1 genes from ten different mammals. Gene 133:197–204.

Ramm SA, Oliver PL, Ponting CP, Stockley P, Emes RD (2008) Sexual selection and the adaptive evolution of mammalian ejaculate proteins. Mol Biol Evol 25:207.

Ramm SA, Parker GA, Stockley P (2005) Sperm competition and the evolution of male reproductive anatomy in rodents. PRSB 272:949-955.

Retief JD, Krajewski C, Westerman M, Dixon GH (1995) The Evolution of Protamine P1 Genes in Dasyurid Marsupials. J Mol Evol 41:549–555.

Retief JD, Winkfein RJ, Dixon GH (1993) Evolution of the monotremes. The sequences of the protamine P1 genes of platypus and echidna. Eur J Biochem 218:457–61.

Rholf FJ (2001) Comparative methods for the analysis of continuous variables:geometric interpretations. Evolution 55:2143–2160.

Roldan ERS (1991) Sperm shape and size: Evolutionary processes in mammals. In: Comparative Spermatology, 20 Years After (Ed. by B. Baccetti), pp. 1001–1010. New York: Raven Press.

Roldan ERS, Gomendio M, Vitullo AD (1992) The evolution of eutherian spermatozoa and underlying selective forces:female selection and sperm competition. Biol Rev 67:551-593.

Rooney AP, Zhang J (1999) Rapid evolution of a primate sperm protein, relaxation of functional constraint or positive Darwinian selection? Mol Biol Evol 16:706.

Rooney AP, Zhang J, Nei M (2000) An unusual form of purifying selection in a sperm protein. Mol Biol Evol 17:278.

Salanti G, Sanderson S, Higgins JTP (2005) Obstacles and opportunities in meta-analysis of genetic association studies. Genetics in Medicine 7:13-20.

Schlicker M, Schnulle V, Schneppel L, Vorob'ev VI, Engel W (1994) Disturbances of nuclear condensation in human spermatozoa: search for mutations in the genes for protamine 1, protamine 2 and transition nuclear protein 1. Hum Reprod 9:2313-2317.

Shapiro MD, Marks ME, Peichel CL, Blackman BK, Nereg KS, Jónsson B, Schluter D, Kingsley DM (2004) Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. Nature 428:717-723.

Shi QX, Roldan ERS (1995) Bicarbonate/CO2 is not required for zona-pellucida-induced or progesterone-induced acrosomal exocytosis of mouse spermatozoa but is essential for capacitation. Biol Reprod 1995; 52:540-546.

Simmons LW (2001) Sperm competition and its evolutionary consequences in the insects. Princeton: Princeton University Press.

Soulsbury CD (2010) Genetic patterns of paternity and testes size in mammals. PLoS One 5:e9581.

Steger K, Failing K, Klonisch T, Behre HM, Manning M, Weidner W, et al (2001) Round spermatids from infertile men exhibit decreased protamine-1 and -2 mRNA. Hum Reprod 16:709–16.

Steger K, Fink L, Failing K, Bohle RM, Kliesch S, Weidner W and Bergmann M (2003) Decreased protamine-1 transcript levels in testis from infertile men. Mol Hum Reprod 9:331–336.

Steppan SJ, Adkins RM, Anderson J (2004) Phylogeny and divergence-date estimates of rapid radiations in muroid rodents based on multiple nuclear genes. Syst Biol 53:533.

Storey BT (2008) Mammalian sperm metabolism: oxygen and sugar, friend and foe. Int J Dev Biol 52:427–437.

Suarez SS (2008) Control of hyperactivation in sperm. Hum Reprod Update 14:647–57.

Suarez SS, Drost M, Redfern K, Gottlieb W (1990) Sperm motility in the oviduct. In: Bavister BD, Cummins J, Roldan ERS (eds), Fertilization in Mammals. Norwell: Serono Symposia 111-124.

Su Y, Wu D, Zhou W, Irwin DM, Zhang Y (2013) Rapid evolution of the mammalian HILS1 gene and the nuclear condensation process during mammalian spermiogenesis. J Genet Genomics 40:55-59.

Swanson WJ, Vacquier VD (2002) The rapid evolution of reproductive proteins. Nat Rev Genet 3:137-144.

Tanaka H, Baba T (2005) Gene expression in spermiogenesis. Cell Mol Life Sci 62:344–354.

Tirosh I, Weinberger A, Bezalel D, Kaganovich M, Barkai N (2008) On the relation between promoter divergence and gene expression evolution. Mol Syst Biol 4:159.

Torgerson DG, Kulathinal RJ, Singh RS (2002) Mammalian sperm proteins are rapidly evolving: evidence of positive selection in functionally diverse genes. Mol Biol Evol 19:1973-1980.

Torregrosa N, Dominguez-Fandos D, Camejo MI, Shirley CR, Meistrich ML, Ballesca JL, Oliva R (2006) Protamine 2 precursors, protamine 1/protamine 2 ratio, DNA integrity and other sperm parameters in infertile patients. Human Reprod 21:2084-2089.

Tourmente M, Gomendio M, Roldan ERS (2011a) Sperm competition and the evolution of sperm design in mammals. BMC Evol Biol 11:12.

Tourmente M, Gomendio M, Roldan ERS (2011b) Mass-specific metabolic rate and sperm competition determine sperm size in marsupial mammals. PLoS One 6:e21244.

Tourmente M, Rowe M, González-Barroso MM, Rial E, Gomendio M, Roldan ERS (2013) Postcopulatory sexual selection increases ATP content in rodent spermatozoa. Evolution 67:1838–1846.

Turner LM, Hoekstra HE (2008) Causes and consequences of the evolution of reproductive proteins. Int J Dev Biol 52:769-780.

Varea Sanchez M (2014) Morfometría geométrica aplicada al estudio evolutivo de los espermatozoides y su relación con determinantes de la fertilidad en roedores. Doctoral Thesis. Universidad Autónoma de Madrid.

Varea Sánchez M, Bastir M, Roldan ERS (2013) Geometric morphometrics of rodent sperm head shape. PLoS One 8:e80607.

Veyrunes F, Dobigny G, Yang F, O'Brien PCM, Catalan J, Robinson TJ, Britton-Davidian J (2006) Phylogenomics of the genus Mus (Rodentia; Muridae): extensive genome repatterning is not restricted to the house mouse. Proc R Soc B 273:2925-2934.

Vicens A, Tourmente M, Roldan ERS (2014)

Structural evolution of CatSper1 in rodents is influenced by sperm competition, with effects on sperm swimming velocity. BMC Evol Biol 14:106

Walters JR, Harrison RG (2011) Decoupling of rapid and adaptive evolution among seminal fluid proteins in Heliconius butterflies with divergent mating systems. Evolution 65:2855–71

Ward WS, Coffey DS (1991) DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. Biol Reprod 44:569–574.

Wassarman P, Chen J, Cohen N, Litscher E, Liu C, Qi H, Williams Z (1999) Structure and function of the mammalian egg zona pellucida. J Exp Zool 285:251–258.

Wray GA (2007) The evolutionary significance of cis-regulatory mutations. Nat Rev Gen 8:206-216

Wu JY, Ribar TJ, Cummings DE, Burton KA, McKnight GS, Means AR (2000) Spermiogenesis and exchange of basic nuclear proteins are impaired in male germ cells lacking Camk4. Nat Genet 25:448-452.

Wyckoff GJ, Wang W, Wu WJ (2000) Rapid evolution of male reproductive genes in the descent of man. Nature 403:304–309.

Yang Z (1998) Likelihood ratio tests for detecting positive selection and application to primate lysozyme evolution. Mol Biol Evol 15:568–573.

Yang Z (2007) PAML 4, phylogenetic analysis by maximum likelihood. Mol Biol Evol 24:1586–1591.

Yang Z, Rannala B (1997) Bayesian phylogenetic inference using DNA sequences, Markov chain Monte Carlo methods. Mol Biol Evol 14:717-724.

Yelick P, Balhorn R, Johnson P, Corzett M, Mazrimas J, Kleene K, Hecht N (1987) Mouse protamine 2 is synthesized as a precursor whereas mouse protamine 1 is not. Mol Cell Biol 7:21-73.

Yu YE, Zhang Y, Unni E, Shirley CR, Deng JM, Russell LD, Weil MM, Behringer RR, Meistrich ML (2000) Abnormal spermatogenesis and reduced fertility in transition nuclear protein 1 deficient mice. PNAS 97:4683-4688.

Zhao M, Shirley CR, Yu YE, Mohapatra B, Zhang Y, Unni E, Deng JM, Arango NA, Weil MM, Russell LD, Behringer RR, Meistrich ML (2001) Targeted disruption of transition protein 2 gene subtly affects spermatogenesis in mice. Mol Cell Biol 21:7243-7255.

CHAPTER 12

ANNEX

ANNEX I: CHAPTER 4: EVOLUTION OF PROTAMINE GENES AND CHANGES IN SPERM HEAD PHENOTYPE IN RODENTS

ANNEX II: CHAPTER 5: SEXUAL SELECTION ON PROTAMINE 1 IN MAMMALS

ANNEX III: CHAPTER 6: SELECTIVE CONSTRAINTS ON PROTAMINE 2 IN PRIMATES AND RODENTS

ANNEX IV: CHAPTER 7: SEXUAL SELECTION ON PROTAMINE AND TRANSITION NUCLEAR PROTEIN EXPRESSION IN MOUSE SPECIES

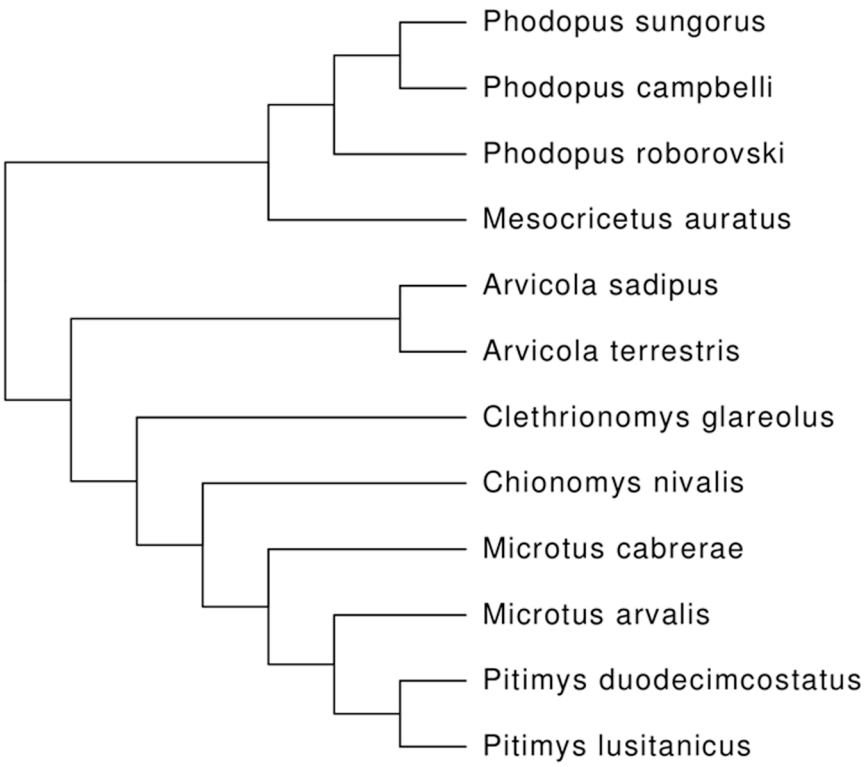
ANNEX V: CHAPTER 8: PROTAMINES AND SPERM HEAD PHENOTYPE: A COMPARATIVE ANALYSIS

ANNEX VI: LIST OF ARTICLES

ANNEX VII: PUBLISHED PAPERS

ANNEX I

CHAPTER 4: EVOLUTION OF PROTAMINE GENES AND CHANGES IN SPERM HEAD PHENOTYPE IN RODENTS



Supplemental Figure S1. Phylogenetic tree of species included in this study.

Supplemental Table 1. Primers used for amplifications.

Primer		Primer sequence
Prm1 (voles and hamsters)	forward	CTCCCGGCCAAGCCAGCACC
	reverse	GGACTTGCTATTCTGTGCAT
Prm2 (hamsters)	forward	ATCCAGGTCAGCTGCAGCC
	reverse	GGCCTGGGGAGGCTTAGTG
Prm2 (voles)	forward	TCATCACCACCAAGAGCAGG
	reverse	GGCCTGGGGAGGCTTAGTG

Supplemental Table S2. Relationships between relative testes mass and measures of head size. CI- and CI+ indicate the confidence intervals for the PGLS regression slope (bold CI values indicate statistical significance), lnL the maximum likelihood estimate of alpha, and alpha the measure of evolutionary constraints acting on phenotypes (COMPARE 4.6b).

	head length	head width
CI-	-0.03	-1.23
CI+	1.02	1.04
lnL	-3.57	-5.12
alpha	2.18	2.09
corr	0.50	-0.05

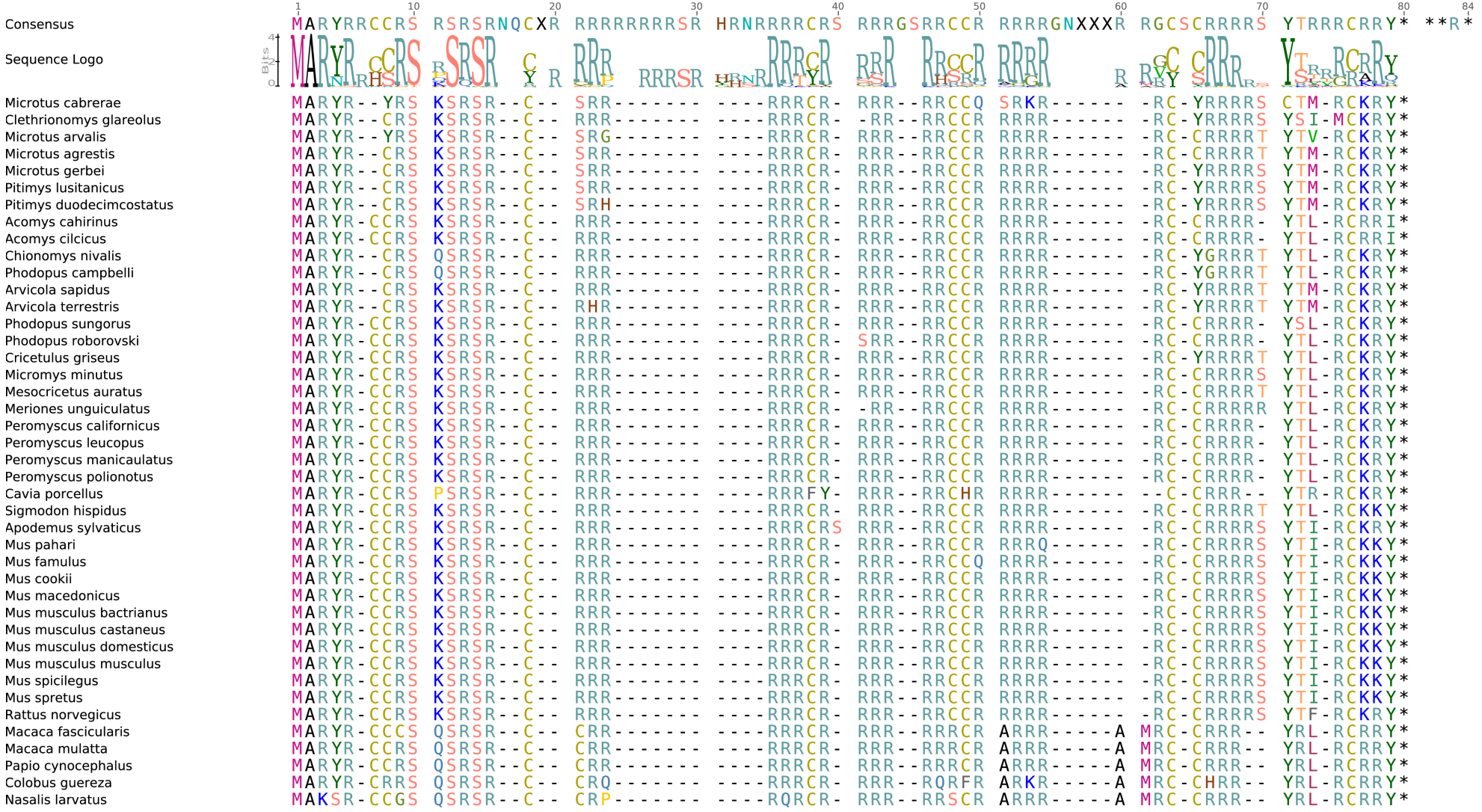
Supplemental Table S3. Relationships between protamine evolutionary rates and measures of head size. CI- and CI+ indicate the confidence intervals for the PGLS regression slope (bold CI values indicate statistical significance), lnL the maximum likelihood estimate of alpha, and alpha the measure of evolutionary constraints acting on phenotypes (COMPARE 4.6b).

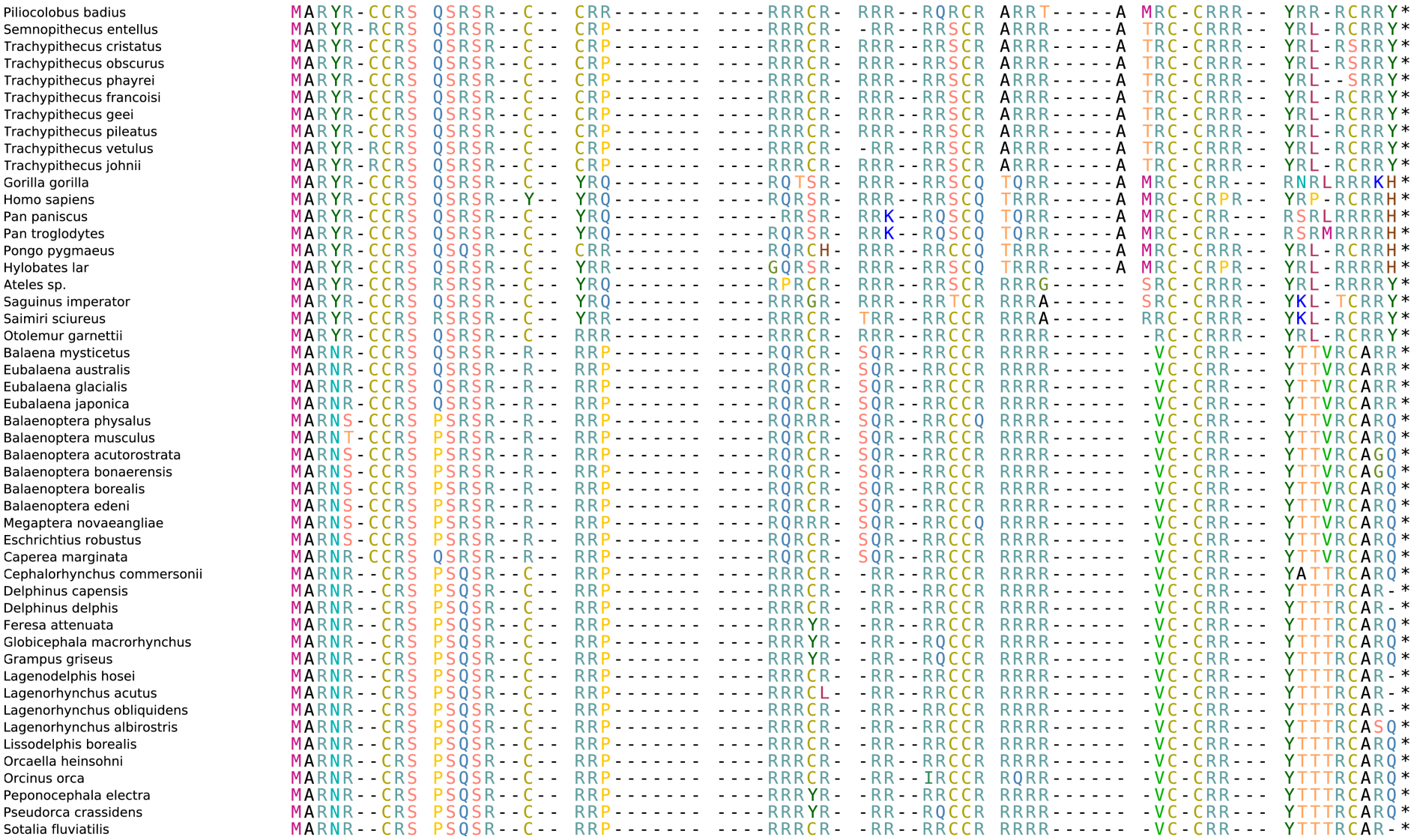
		head length	head width
Protamine 1	CI-	-0.15	**0.10**
	CI+	0.13	**0.49**
	lnL	12.12	15.68
	alpha	1.18	1.70
	corr	-0.06	0.68
cleaved *Protamine 2*	CI-	-0.09	-0.06
	CI+	0.01	0.15
	lnL	24.71	23.51
	alpha	1.89	3.03
	corr	-0.48	0.27
mature *Protamine 2*	CI-	-0.30	-0.48
	CI+	0.09	0.28
	lnL	7.65	7.14
	alpha	0.57	0.50
	corr	-0.33	-0.15

ANNEX II

CHAPTER 5: SEXUAL SELECTION ON PROTAMINE 1 IN MAMMALS

Figure S1. Multiple sequence alignment for all 237 included mammal species based on muscle algorithm (implemented in Geneious 5.5.9.)

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Trachypithecus cristatus MARYR-CCRS QSRSR--C-- CRP------- ----RRRCR- RRR--RRSCR ARRR-----A TRC-CRRR-- YRL-RSRRY*
Trachypithecus obscurus MARYR-CCRS QSRSR--C-- CRP------- ----RRRCR- RRR--RRSCR ARRR-----A TRC-CRRR-- YRL-RSRRY*
Trachypithecus phayrei MARYR-CCRS QSRSR--C-- CRP------- ----RRRCR- RRR--RRSCR ARRR-----A TRC-CRRR-- YRL--SRRY*
Trachypithecus francoisi MARYR-CCRS QSRSR--C-- CRP------- ----RRRCR- RRR--RRSCR ARRR-----A TRC-CRRR-- YRL-RCRRY*
Trachypithecus geei MARYR-CCRS QSRSR--C-- CRP------- ----RRRCR- RRR--RRSCR ARRR-----A TRC-CRRR-- YRL-RCRRY*
Trachypithecus pileatus MARYR-CCRS QSRSR--C-- CRP------- ----RRRCR- RRR--RRSCR ARRR-----A TRC-CRRR-- YRL-RCRRY*
Trachypithecus vetulus MARYR-RCRS QSRSR--C-- CRP------- ----RRRCR- -RR--RRSCR ARRR-----A TRC-CRRR-- YRL-RCRRY*
Trachypithecus johnii MARYR-RCRS QSRSR--C-- CRP------- ----RRRCR- RRR--RRSCR ARRR-----A TRC-CRRR-- YRL-RCRRY*
Gorilla gorilla MARYR-CCRS QSRSR--C-- YRQ------- ----RQTSR- RRR--RRSCQ TQRR-----A MRC-CRR--- RNRLRRRKH*
Homo sapiens MARYR-CCRS QSRSR--Y-- YRQ------- ----RQRSR- RRR--RRSCQ TRRR-----A MRC-CRPR-- YRP-RCRRH*
Pan paniscus MARYR-CCRS QSRSR--C-- YRQ------- -----RRSR- RRK--RQSCQ TQRR-----A MRC-CRR--- RSRLRRRRH*
Pan troglodytes MARYR-CCRS QSRSR--C-- YRQ------- -----RQRSR- RRK--RQSCQ TQRR-----A MRC-CRR--- RSRMRRRRH*
Pongo pygmaeus MARYR-CCRS QSQSR--C-- CRR------- ----RQRCH- RRR--RRCCQ TRRR-----A MRC-CRRR-- YRL-RCRRH*
Hylobates lar MARYR-CCRS QSRSR--C-- YRR------- ----GQRSR- RRR--RRSCQ TRRR-----A MRC-CRPR-- YRL-RRRRH*
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Saguinus imperator MARYR-CCRS QSRSR--C-- YRQ------- ----RRRGR- RRR--RRTCR RRRA------ SRC-CRRR-- YKL-TCRRY*
Saimiri sciureus MARYR-CCRS RSRSR--C-- YRR------- ----RRRCR- TRR--RRCCR RRRA------ RRC-CRRR-- YKL-RCRRY*
Otolemur garnettii MARYR-CCRS QSRSR--C-- RRR------- ----RRRCR- RRR--RRCCR RRRR------ -RC-CRRR-- YRL-RCRRY*
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Eubalaena australis MARNR-CCRS QSRSR--R-- RRP------- ----RQRCR- SQR--RRCCR RRRR------ -VC-CRR--- YTTVRCARR*
Eubalaena glacialis MARNR-CCRS QSRSR--R-- RRP------- ----RQRCR- SQR--RRCCR RRRR------ -VC-CRR--- YTTVRCARR*
Eubalaena japonica MARNR-CCRS QSRSR--R-- RRP------- ----RQRCR- SQR--RRCCR RRRR------ -VC-CRR--- YTTVRCARR*
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Balaenoptera bonaerensis MARNS-CCRS PSRSR--R-- RRP------- ----RQRCR- SQR--RRCCR RRRR------ -VC-CRR--- YTTVRCAGQ*
Balaenoptera borealis MARNS-CCRS PSRSR--R-- RRP------- ----RQRCR- SQR--RRCCR RRRR------ -VC-CRR--- YTTVRCARQ*
Balaenoptera edeni MARNS-CCRS PSRSR--R-- RRP------- ----RQRCR- SQR--RRCCR RRRR------ -VC-CRR--- YTTVRCARQ*
Megaptera novaeangliae MARNS-CCRS PSRSR--R-- RRP------- ----RQRRR- SQR--RRCCQ RRRR------ -VC-CRR--- YTTVRCARQ*
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Delphinus delphis MARNR--CRS PSQSR--C-- RRP------- ----RRRCR- -RR--RRCCR RRRR------ -VC-CRR--- YTTTRCAR-*
Feresa attenuata MARNR--CRS PSQSR--C-- RRP------- ----RRRYR- -RR--RRCCR RRRR------ -VC-CRR--- YTTTRCARQ*
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Lagenodelphis hosei MARNR--CRS PSQSR--C-- RRP------- ----RRRCR- -RR--RRCCR RRRR------ -VC-CRR--- YTTTRCAR-*
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Lagenorhynchus obliquidens MARNR--CRS PSQSR--C-- RRP------- ----RRRCR- -RR--RRCCR RRRR------ -VC-CRR--- YTTTRCAR-*
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Lissodelphis borealis MARNR--CRS PSQSR--C-- RRP------- ----RRRCR- -RR--RRCCR RRRR------ -VC-CRR--- YTTTRCARQ*
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Orcinus orca MARNR--CRS PSQSR--C-- RRP------- ----RRRCR- -RR--IRCCR RQRR------ -VC-CRR--- YTTTRCARQ*
Peponocephala electra MARNR--CRS PSQSR--C-- RRP------- ----RRRYR- -RR--RRCCR RRRR------ -VC-CRR--- YTTTRCARQ*
Pseudorca crassidens MARNR--CRS PSQSR--C-- RRP------- ----RRRYR- -RR--RQCCR RRRR------ -VC-CRR--- YTTTRCARQ*
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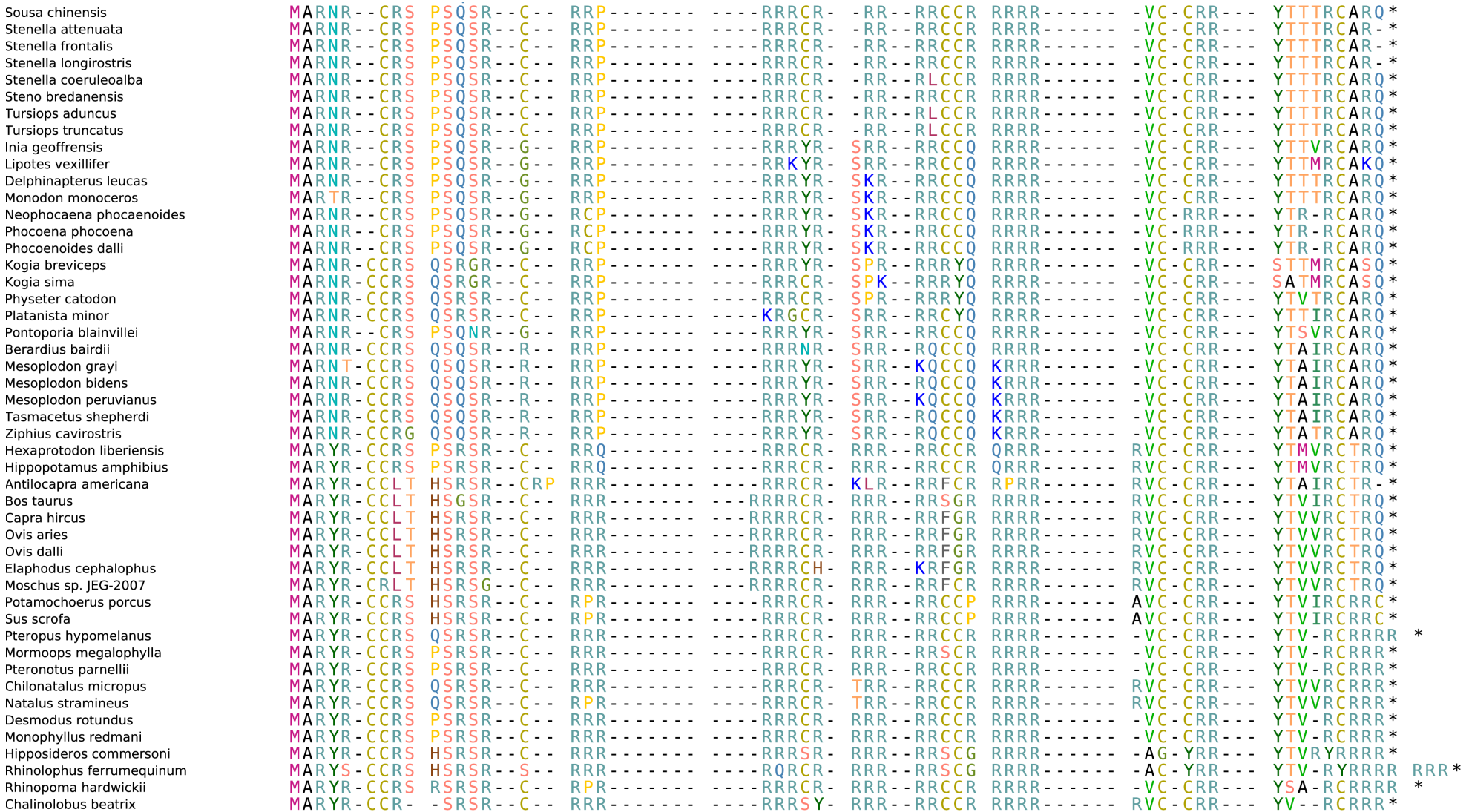
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Delphinapterus leucas MARNR--CRS PSQSR--G-- RRP------- ----RRRYR- SKR--RRCCQ RRRR------ -VC-CRR--- YTTTRCARQ*
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Physeter catodon MARNR-CCRS QSRSR--C-- RRP------- ----RRRCR- SPR--RRRYQ RRRR------ -VC-CRR--- YTVTRCARQ*
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Pontoporia blainvillei MARNR--CRS PSQNR--G-- RRP------- ----RRRYR- SRR--RRCCQ RRRR------ -VC-CRR--- YTSVRCARQ*
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Tasmacetus shepherdi MARNR-CCRS QSQSR--R-- RRP------- ----RRRYR- SRR--RQCCQ KRRR------ -VC-CRR--- YTAIRCARQ*
Ziphius cavirostris MARNR-CCRG QSQSR--R-- RRP------- ----RRRYR- SRR--RQCCQ KRRR------ -VC-CRR--- YTATRCARQ*
Hexaprotodon liberiensis MARYR-CCRS PSRSR--C-- RRQ------- ----RRRCR- RRR--RRCCR QRRR------ RVC-CRR--- YTMVRCTRQ*
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Antilocapra americana MARYR-CCLT HSRSR--CRP RRR------- ----RRRCR- KLR--RRFCR RPRR------ RVC-CRR--- YTAIRCTR-*
Bos taurus MARYR-CCLT HSGSR--C-- RRR------- ---RRRRCR- RRR--RRSGR RRRR------ RVC-CRR--- YTVIRCTRQ*
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Ovis aries MARYR-CCLT HSRSR--C-- RRR------- ---RRRRCR- RRR--RRFGR RRRR------ RVC-CRR--- YTVVRCTRQ*
Ovis dalli MARYR-CCLT HSRSR--C-- RRR------- ---RRRRCR- RRR--RRFGR RRRR------ RVC-CRR--- YTVVRCTRQ*
Elaphodus cephalophus MARYR-CCLT HSRSR--C-- RRR------- ---RRRRCH- RRR--KRFGR RRRR------ RVC-CRR--- YTVVRCTRQ*
Moschus sp. JEG-2007 MARYR-CRLT HSRSG--C-- RRR------- ---RRRRCR- RRR--RRFCR RRRR------ RVC-CRR--- YTVVRCTRQ*
Potamochoerus porcus MARYR-CCRS HSRSR--C-- RPR------- ----RRRCR- RRR--RRCCP RRRR------ AVC-CRR--- YTVIRCRRC*
Sus scrofa MARYR-CCRS HSRSR--C-- RPR------- ----RRRCR- RRR--RRCCP RRRR------ AVC-CRR--- YTVIRCRRC*
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Hipposideros commersoni MARYR-CCRS HSRSR--C-- RRR------- ----RRRSR- RRR--RRSCG RRRR------ -AG-YRR--- YTVRYRRRR*
Rhinolophus ferrumequinum MARYS-CCRS HSRSR--S-- RRR------- ----RQRCR- RRR--RRSCG RRRR------ -AC-YRR--- YTV-RYRRRR RRR*
Rhinopoma hardwickii MARYR-CCRS RSRSR--C-- RPR------- ----RRRCR- RRR--RRCCR RRRR------ -VC-CRR--- YSA-RCRRRR *
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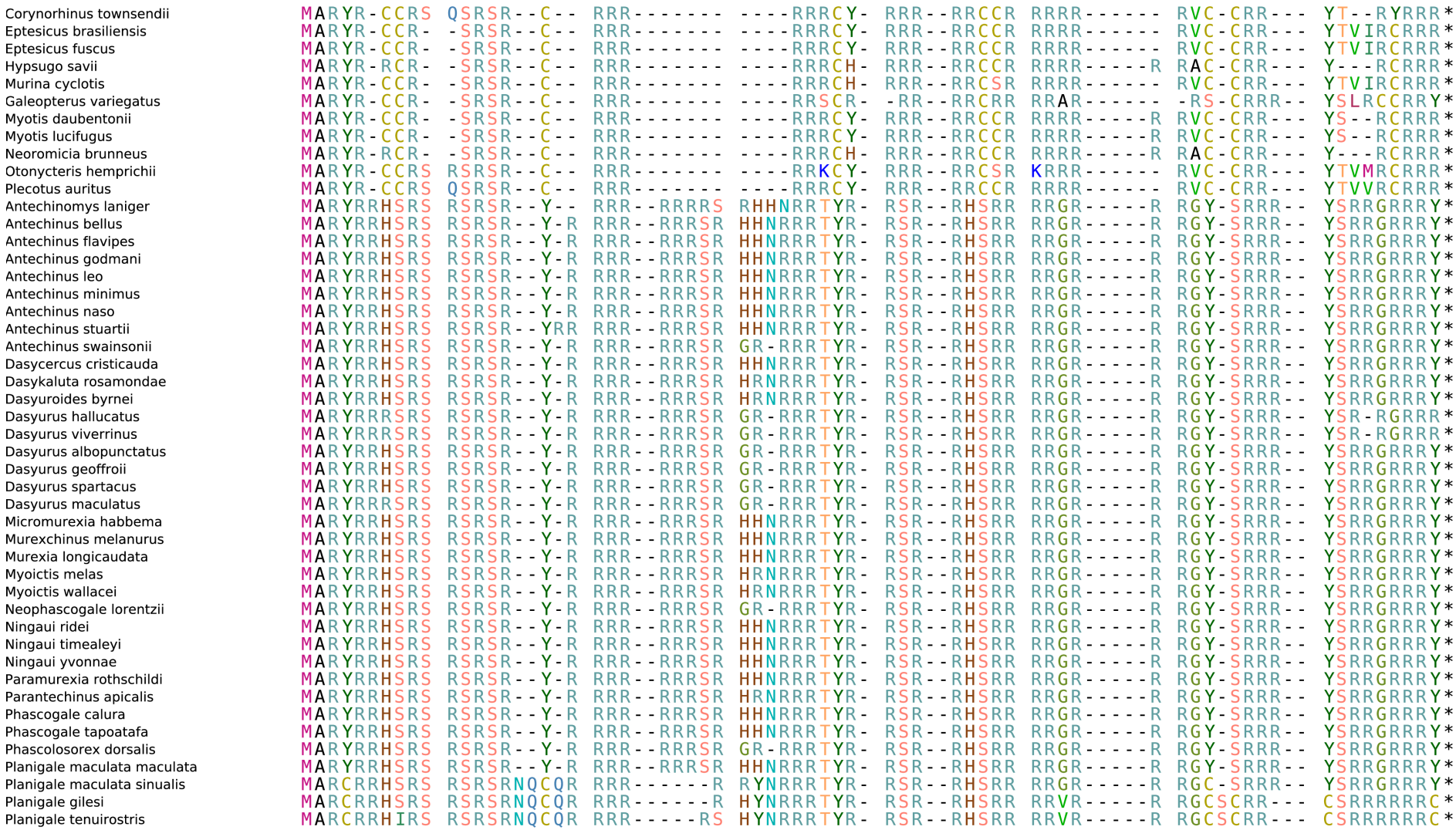
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Neoromicia brunneus
Otonycteris hemprichii
Plecotus auritus
Antechinomys laniger
Antechinus bellus
Antechinus flavipes
Antechinus godmani
Antechinus leo
Antechinus minimus
Antechinus naso
Antechinus stuartii
Antechinus swainsonii
Dasycercus cristicauda
Dasykaluta rosamondae
Dasyuroides byrnei
Dasyurus hallucatus
Dasyurus viverrinus
Dasyurus albopunctatus
Dasyurus geoffroii
Dasyurus spartacus
Dasyurus maculatus
Micromurexia habbema
Murexchinus melanurus
Murexia longicaudata
Myoictis melas
Myoictis wallacei
Neophascogale lorentzii
Ningaui ridei
Ningaui timealeyi
Ningaui yvonnae
Paramurexia rothschildi
Parantechinus apicalis
Phascogale calura
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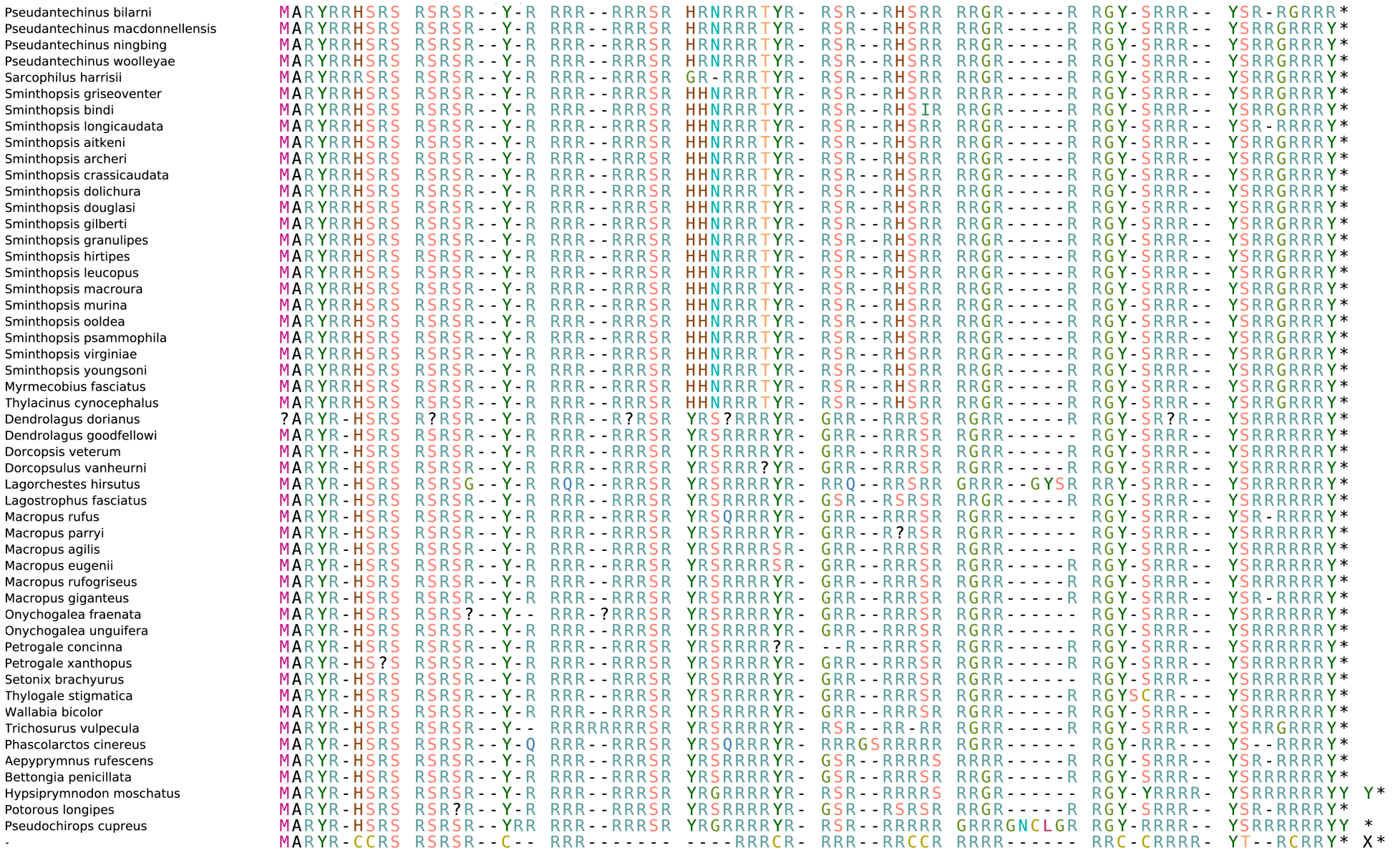
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Pseudantechinus ningbing
Pseudantechinus woolleyae
Sarcophilus harrisii
Sminthopsis griseoventer
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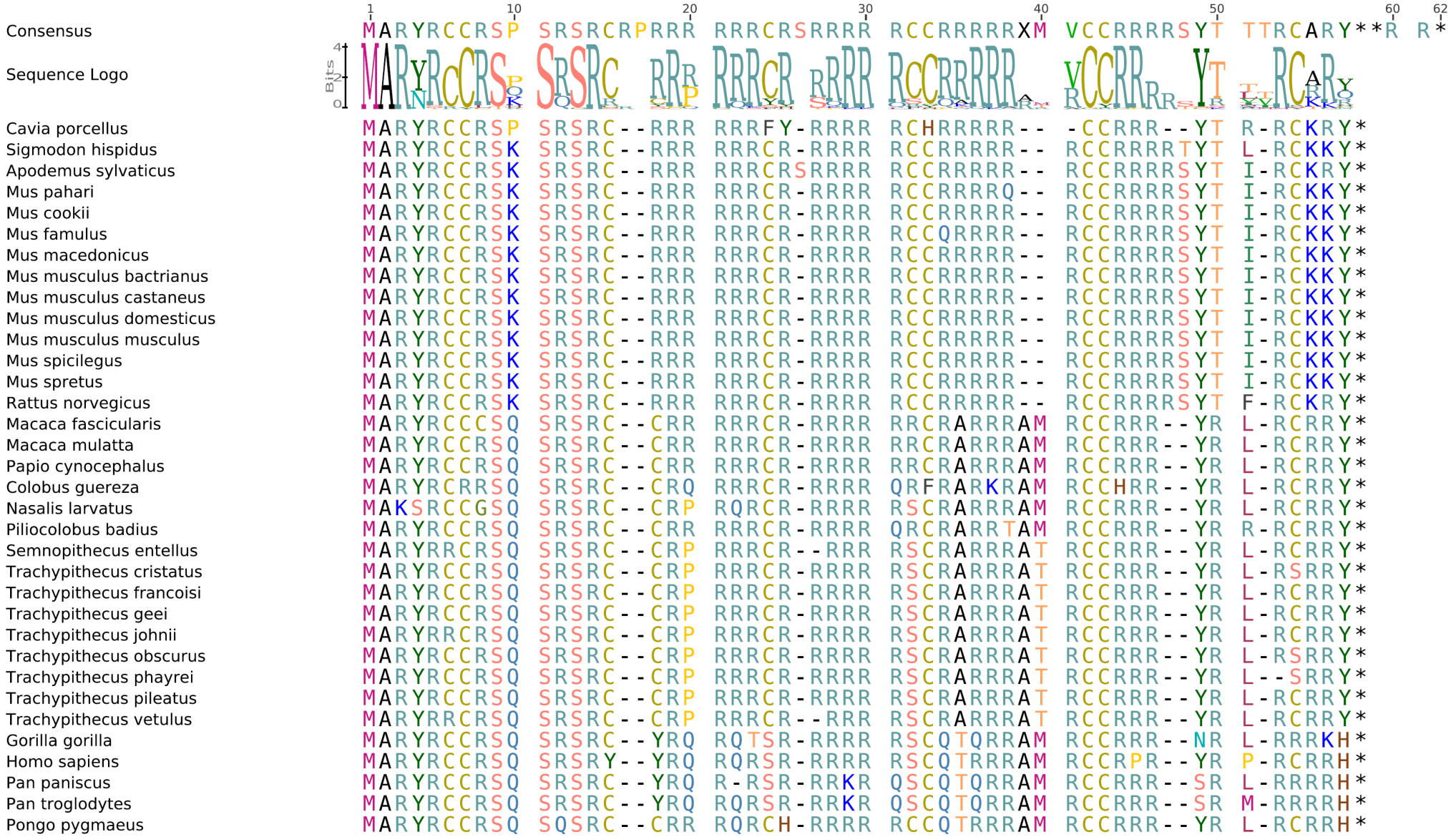
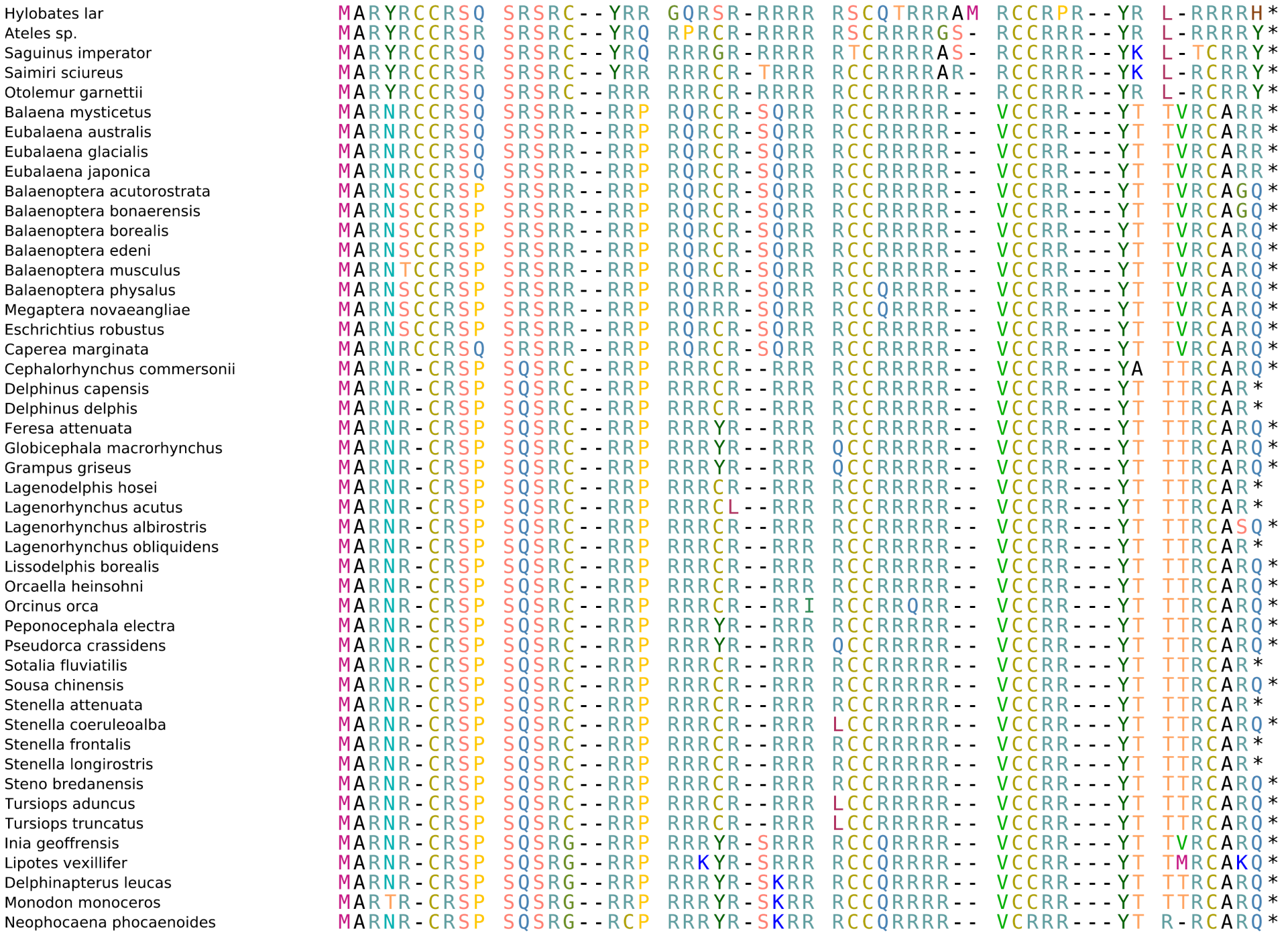
Sminthopsis longicaudata
Sminthopsis aitkeni
Sminthopsis archeri
Sminthopsis crassicaudata
Sminthopsis dolichura
Sminthopsis douglasi
Sminthopsis gilberti
Sminthopsis granulipes
Sminthopsis hirtipes
Sminthopsis leucopus
Sminthopsis macroura
Sminthopsis murina
Sminthopsis ooldea
Sminthopsis psammophila
Sminthopsis virginiae
Sminthopsis youngsoni
Myrmecobius fasciatus
Thylacinus cynocephalus
Dendrolagus dorianus
Dendrolagus goodfellowi
Dorcopsis veterum
Dorcopsulus vanheurni
Lagorchestes hirsutus
Lagostrophus fasciatus
Macropus rufus
Macropus parryi
Macropus agilis
Macropus eugenii
Macropus rufogriseus
Macropus giganteus
Onychogalea fraenata
Onychogalea unguifera
Petrogale concinna
Petrogale xanthopus
Setonix brachyurus
Thylogale stigmatica
Wallabia bicolor
Trichosurus vulpecula
Phascolarctos cinereus
Aepyprymnus rufescens
Bettongia penicillata
Hypsiprymnodon moschatus
Potorous longipes
Pseudochirops cupreus
-

Figure S2. Multiple sequence alignment for included eutherian species based on muscle algorithm (implemented in Geneious 5.5.9.)

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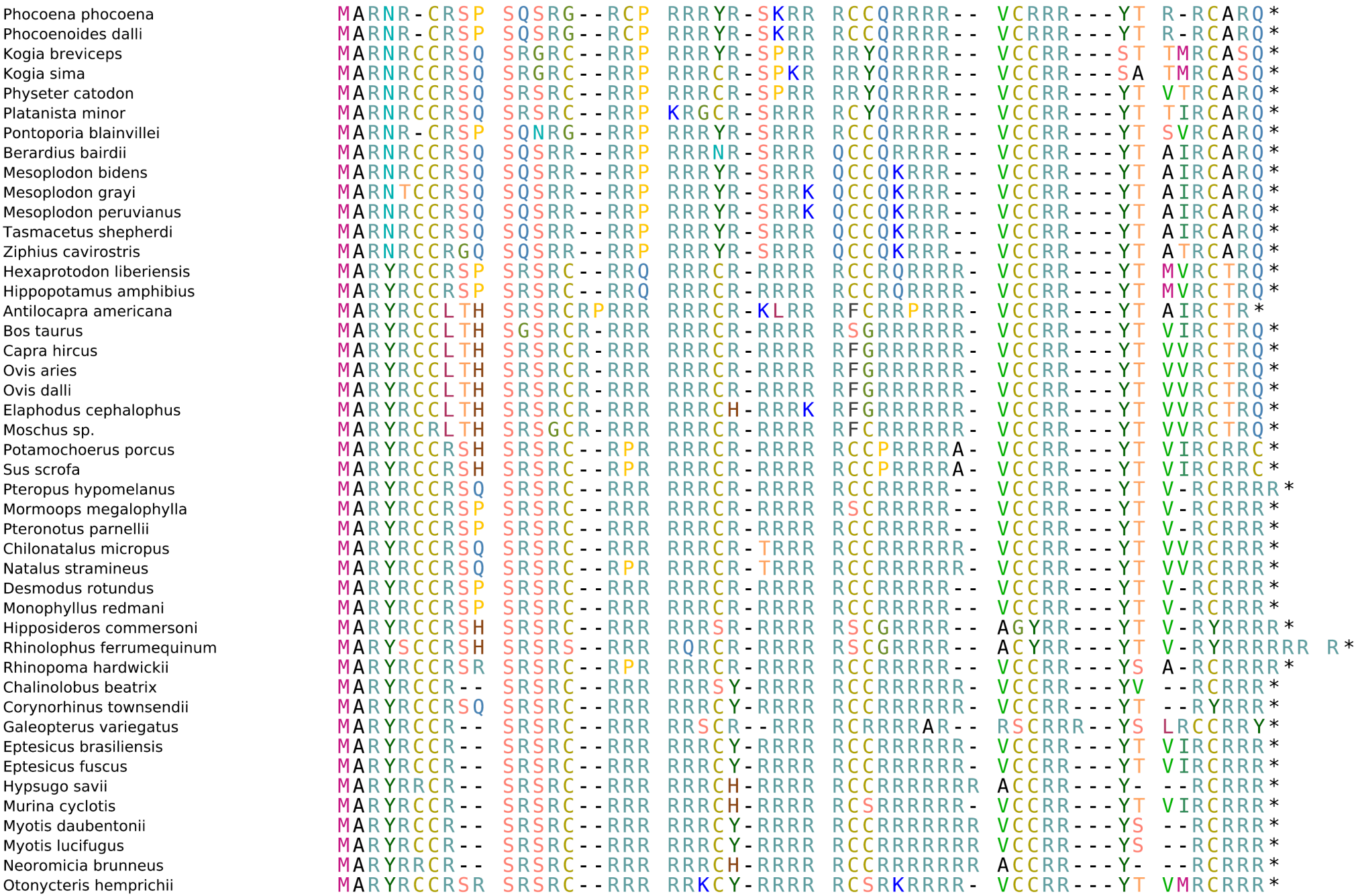
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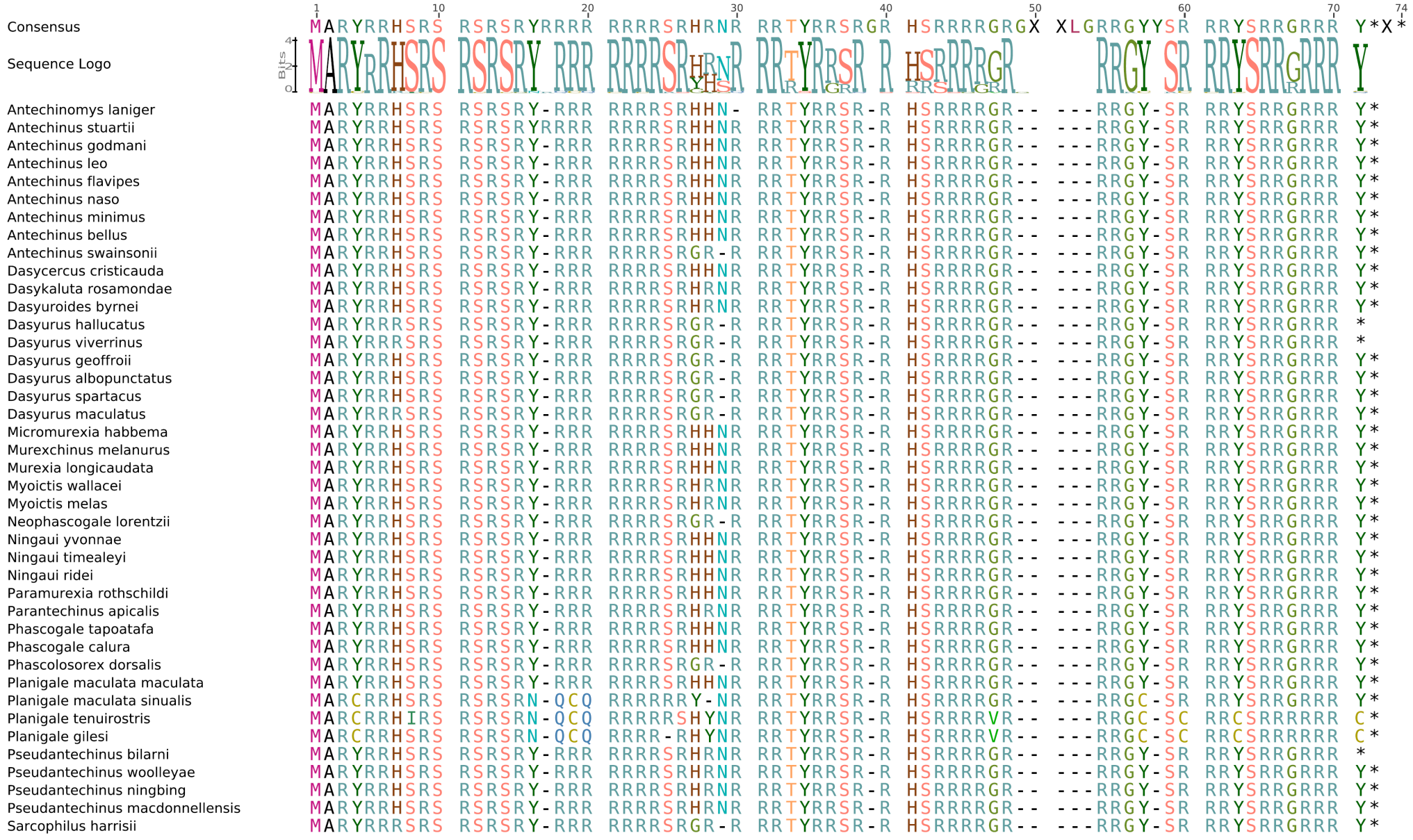
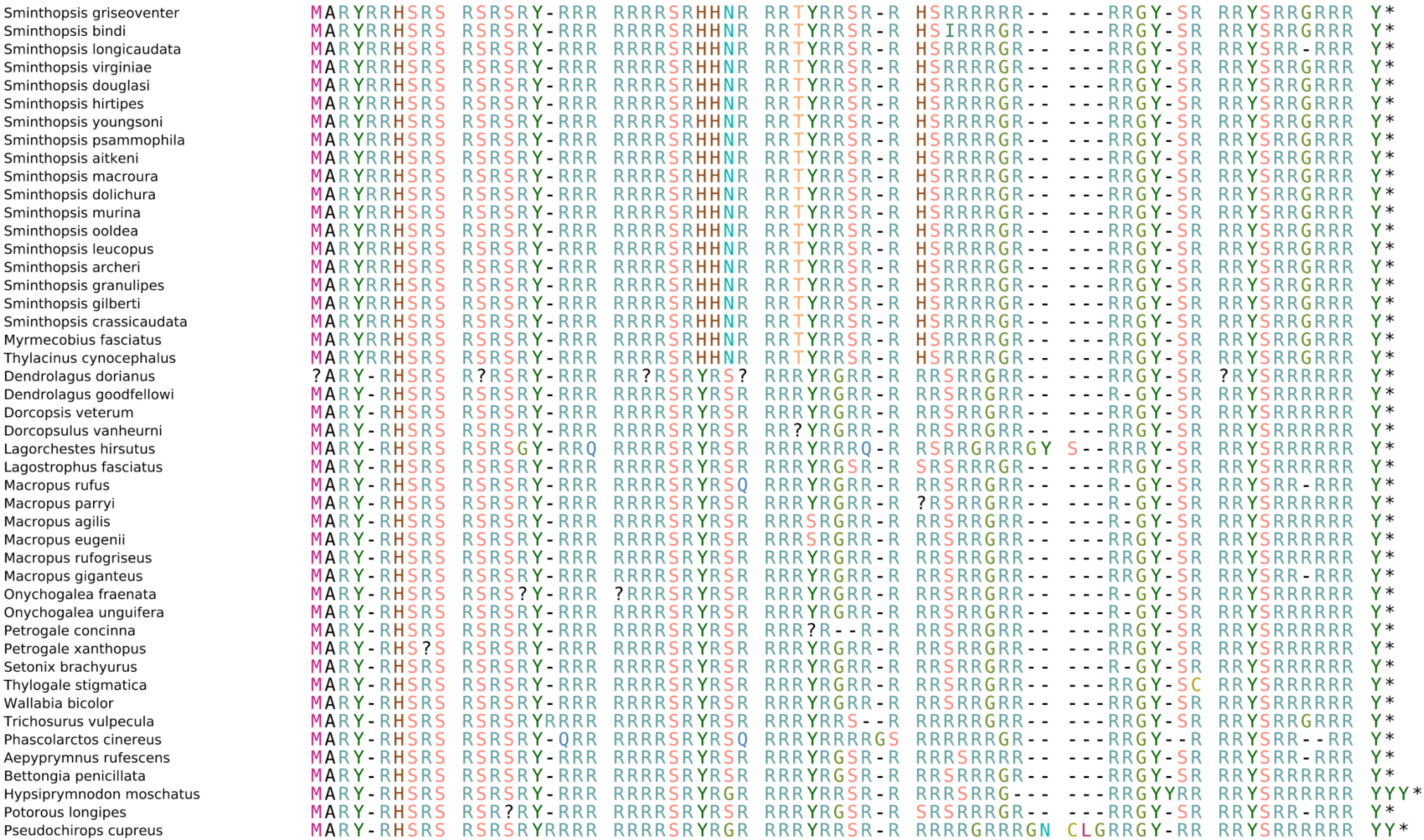
Figure S3. Multiple sequence alignment for included metatherian species based on muscle algorithm (implemented in Geneious 5.5.9.)

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Onychogalea unguifera MARY-RHSRS RSRSRY-RRR RRRRSRYRSR RRRYRGRR-R RRSRRGRR-- ---R-GY-SR RRYSRRRRRR Y*
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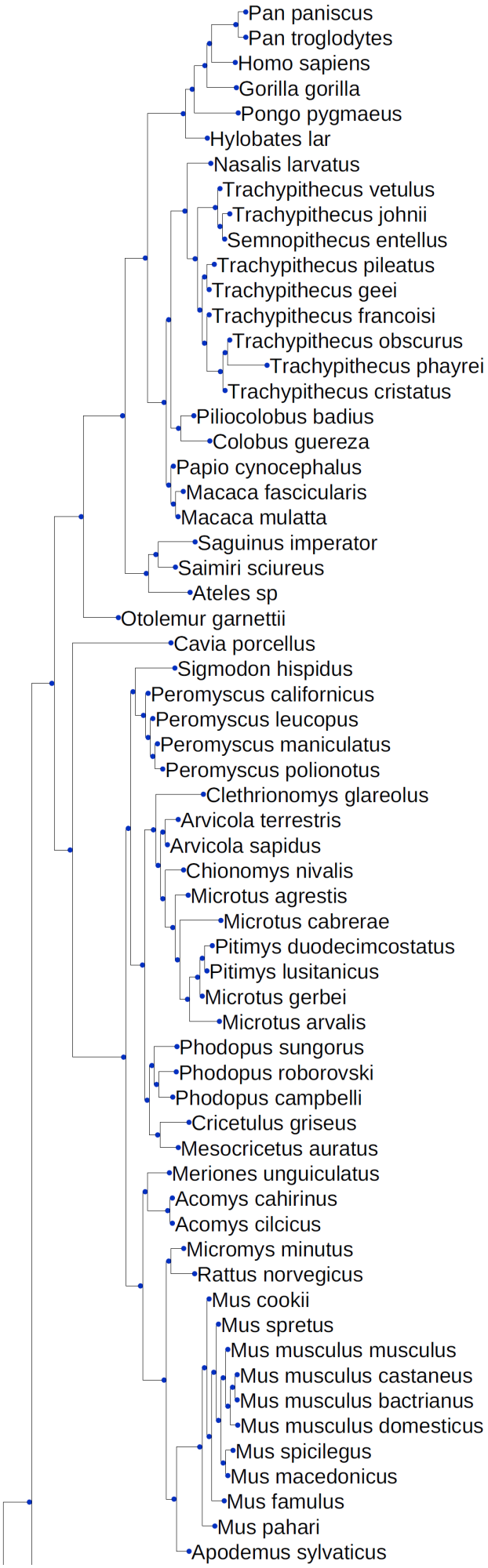
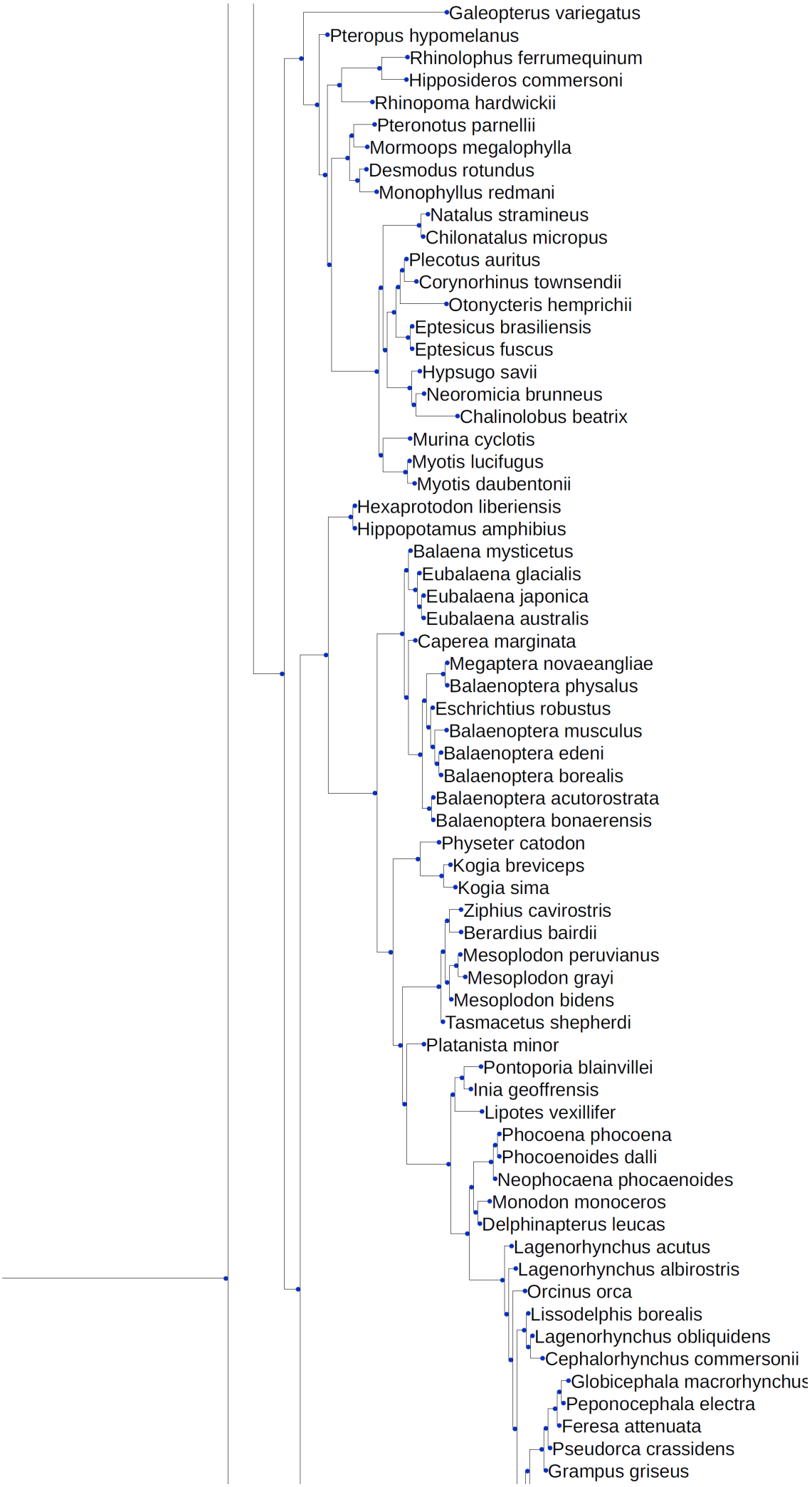
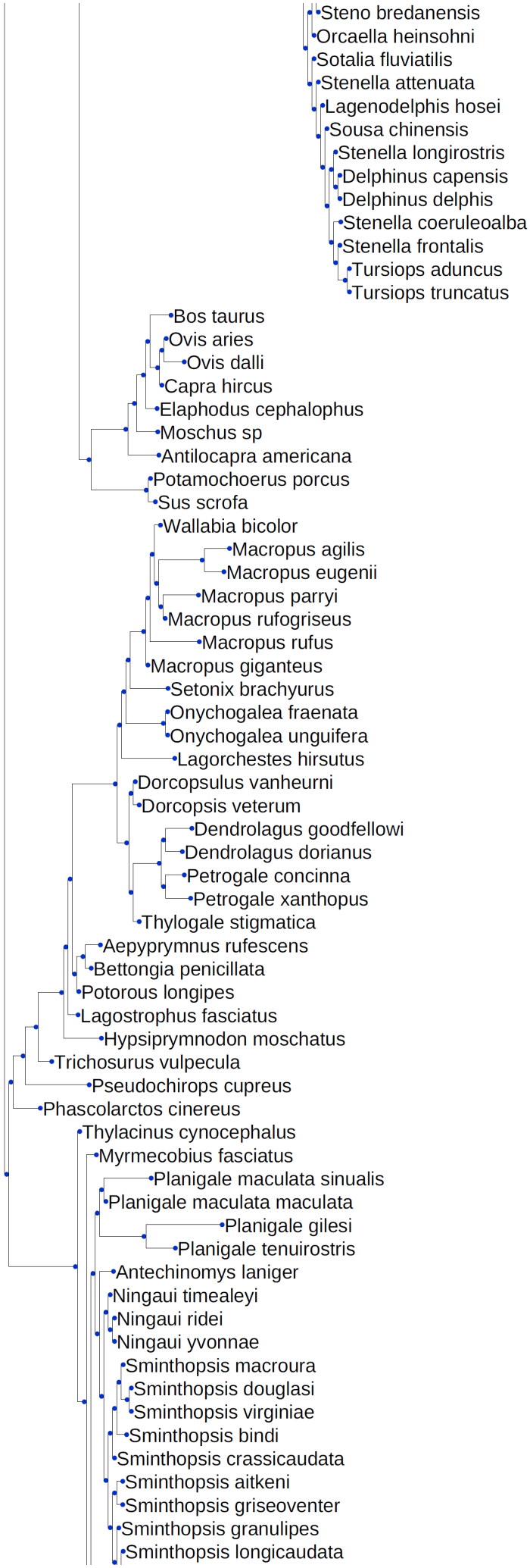
Wallabia bicolor MARY-RHSRS RSRSRY-RRR RRRRSRYRSR RRRYRGRR-R RRSRRGRR-- ---RRGY-SR RRYSRRRRRR Y*
Trichosurus vulpecula MARY-RHSRS RSRSRYRRRR RRRRSRYRSR RRRYRRS--R RRRRRGRR-- ---RRGY-SR RRYSRRGRRR Y*
Phascolarctos cinereus MARY-RHSRS RSRSRY-QRR RRRRSRYRSQ RRRYRRRRGS RRRRRRGR-- ---RRGY--R RRYSRR--RR Y*
Aepyprymnus rufescens MARY-RHSRS RSRSRY-RRR RRRRSRYRSR RRRYRGSR-R RRRSRRRR-- ---RRGY-SR RRYSRR-RRR Y*
Bettongia penicillata MARY-RHSRS RSRSRY-RRR RRRRSRYRSR RRRYRGSR-R RRSRRRGR-- ---RRGY-SR RRYSRRRRRR Y*
Hypsiprymnodon moschatus MARY-RHSRS RSRSRY-RRR RRRRSRYRGR RRRYRRSR-R RRRSRRG--- ---RRGYYRR RRYSRRRRRR YYY*
Potorous longipes MARY-RHSRS RSR?RY-RRR RRRRSRYRSR RRRYRGSR-R SRSRRRGR-- ---RRGY-SR RRYSRR-RRR Y*
Pseudochirops cupreus MARY-RHSRS RSRSRYRRRR RRRRSRYRGR RRRYRRSR-R RRRRGRRRGN CLGRRGY-RR RRYSRRRRRR YY*

Figure S4. Phylogenetic tree constructed as consensus of phylogenetic data available in literature. Branch lengths represent protamine 1 nucleotide substitution rates (PAML4, M0) . References below.




Galeopterus variegatus
Pteropus hypomelanus
Rhinolophus ferrumequinum
Hipposideros commersoni
Rhinopoma hardwickii
Pteronotus parnellii
Mormoops megalophylla
Desmodus rotundus
Monophyllus redmani
Natalus stramineus
Chilonatalus micropus
Plecotus auritus
Corynorhinus townsendii
Otonycteris hemprichii
Eptesicus brasiliensis
Eptesicus fuscus
Hypsugo savii
Neoromicia brunneus
Chalinolobus beatrix
Murina cyclotis
Myotis lucifugus
Myotis daubentonii
Hexaprotodon liberiensis
Hippopotamus amphibius
Balaena mysticetus
Eubalaena glacialis
Eubalaena japonica
Eubalaena australis
Caperea marginata
Megaptera novaeangliae
Balaenoptera physalus
Eschrichtius robustus
Balaenoptera musculus
Balaenoptera edeni
Balaenoptera borealis
Balaenoptera acutorostrata
Balaenoptera bonaerensis
Physeter catodon
Kogia breviceps
Kogia sima
Ziphius cavirostris
Berardius bairdii
Mesoplodon peruvianus
Mesoplodon grayi
Mesoplodon bidens
Tasmacetus shepherdi
Platanista minor
Pontoporia blainvillei
Inia geoffrensis
Lipotes vexillifer
Phocoena phocoena
Phocoenoides dalli
Neophocaena phocaenoides
Monodon monoceros
Delphinapterus leucas
Lagenorhynchus acutus
Lagenorhynchus albirostris
Orcinus orca
Lissodelphis borealis
Lagenorhynchus obliquidens
Cephalorhynchus commersonii
Globicephala macrorhynchus
Peponocephala electra
Feresa attenuata
Pseudorca crassidens
Grampus griseus


Steno bredanensis
Orcaella heinsohni
Sotalia fluviatilis
Stenella attenuata
Lagenodelphis hosei
Sousa chinensis
Stenella longirostris
Delphinus capensis
Delphinus delphis
Stenella coeruleoalba
Stenella frontalis
Tursiops aduncus
Tursiops truncatus
Bos taurus
Ovis aries
Ovis dalli
Capra hircus
Elaphodus cephalophus
Moschus sp
Antilocapra americana
Potamochoerus porcus
Sus scrofa
Wallabia bicolor
Macropus agilis
Macropus eugenii
Macropus parryi
Macropus rufogriseus
Macropus rufus
Macropus giganteus
Setonix brachyurus
Onychogalea fraenata
Onychogalea unguifera
Lagorchestes hirsutus
Dorcopsulus vanheurni
Dorcopsis veterum
Dendrolagus goodfellowi
Dendrolagus dorianus
Petrogale concinna
Petrogale xanthopus
Thylogale stigmatica
Aepyprymnus rufescens
Bettongia penicillata
Potorous longipes
Lagostrophus fasciatus
Hypsiprymnodon moschatus
Trichosurus vulpecula
Pseudochirops cupreus
Phascolarctos cinereus
Thylacinus cynocephalus
Myrmecobius fasciatus
Planigale maculata sinualis
Planigale maculata maculata
Planigale gilesi
Planigale tenuirostris
Antechinomys laniger
Ningaui timealeyi
Ningaui ridei
Ningaui yvonnae
Sminthopsis macroura
Sminthopsis douglasi
Sminthopsis virginiae
Sminthopsis bindi
Sminthopsis crassicaudata
Sminthopsis aitkeni
Sminthopsis griseoventer
Sminthopsis granulipes
Sminthopsis longicaudata


Sminthopsis hirtipes
Sminthopsis psammophila
Sminthopsis youngsoni
Sminthopsis ooldea
Sminthopsis archeri
Sminthopsis dolichura
Sminthopsis gilberti
Sminthopsis leucopus
Sminthopsis murina
Phascogale tapoatafa
Phascogale calura
Antechinus naso
Murexia longicaudata
Murexchinus melanurus
Paramurexia rothschildi
Micromurexia habbema
Antechinus swainsonii
Antechinus minimus
Antechinus godmani
Antechinus stuartii
Antechinus flavipes
Antechinus leo
Antechinus bellus
Dasykaluta rosamondae
Myoictis melas
Myoictis wallacei
Pseudantechinus ningbing
Pseudantechinus bilarni
Parantechinus apicalis
Pseudantechinus macdonnellensis
Dasycercus cristicauda
Pseudantechinus woolleyae
Neophascogale lorentzii
Phascolosorex dorsalis
Dasyuroides byrnei
Sarcophilus harrisii
Dasyurus hallucatus
Dasyurus maculatus
Dasyurus viverrinus
Dasyurus albopunctatus
Dasyurus geoffroii
Dasyurus spartacus
0.31

References for Suppl. Fig. S4

Adkins RM, Walton AH, Honeycutt RL (2003) Higher-level systematics of rodents and divergence time estimates based on two congruent nuclear genes. Mol Phylogenet Evol26:409–420.

Agnarsson I, May-Collado LJ (2008) The phylogeny of Cetartiodactyla: The importance of dense taxon sampling, missing data, and the remarkable promise of cytochrome b toprovide reliable species-level phylogenies. Mol Phylogenet Evol 48:964–985.

Agnarsson I, Zambrana-Torrelio CM, Flores-Saldana NP, May-Collado LJ (2011) A time-calibrated species-level phylogeny of bats (Chiroptera, Mammalia). PLOS Currents Tree of Life. 2011 Feb 4. Edition 1

Almeida FC, Bonvicino CR, Cordeiro-Estrela P (2007). Phylogeny and temporal diversification of Calomys (Rodentia, Sigmodontinae): Implications for the biogeographyof an endemic genus of the open/dry biomes of South America. Mol Phylogenet Evol42:449–466.

Álvarez A, Perez SI, Verzi DH (2011) Ecological and phylogenetic influence on mandible shape variation of South American caviomorph rodents (Rodentia: Hystricomorpha). Biol. J. Linn. Soc. 102:828-837.

Baena A, Mootnick AR, Falvo JV, Tsytskova AV, Ligeiro F, Diop OM, Brieva C, GagneuxP, O'Brien SJ, Ryder OA, Goldfeld AE (2005) Primate TNF promoters reveal markers ofphylogeny and evolution of innate immunity. PLoS One 2:e621.

Beck RM, Bininda-Emonds OR, Cardillo M, Liu FR, Purvis A (2006) A higher-level MRP supertree of placental mammals. BMC Evol Biol 6:93–107.

Bininda-Emonds OR, Cardillo M, Jones KE, MacPhee RDE, Beck RMD, Greyner R, PriceSA, Vos RA, Gittleman JL, Purvis A (2007) The delayed rise of present-day mammals.Nature 446:507–512.

Bininda-Emonds ORP, Cardillo M, Jones KE, MacPhee RDE, Beck RMD, Grenyer R, Price SA, Vos RA, Gittleman JL, Purvis A (2007) The delayed rise of present-day mammals. Nature 446:507-512.

Böhm M, Mayhew PJ (2005) Historical biogeography and the evolution of the latitudinal gradient of species richness in the Papionini (Primata: Cercopithecidae). Biol J Linn Soc-Lond 85:235–246.

Borges BN, Paiva TS, Harada ML (2008) Evolution of the SEC1 gene in New World monkey lineages (Primates, Platyrrhini). Genet Mol Res 7:663–678.

Bradley RD, Durish ND, Rogers DS, Miller JR, Engstrom MD, Kilpatrick CW (2007) Toward a molecular phylogeny for Peromyscus: evidence from mitochondrial cytochrome-b sequences. J Mammal 88:1146–1159.

Cardillo M, Bininda-Emonds ORP, Boakes E, Purvis A (2004) A species-level phylogenetic supertree of marsupials. J Zool 264:11-31.

Conroy CJ, Cook JA (2000) Molecular systematics of a holartic rodent (Microtus: Muridae). J Mammal 81:344–359.

Flynn JJ, Finarelli JA, Zehr S, Hsu J, Nedbal MA (2005) Molecular phylogeny of the Carnivora (Mammalia): assessing the impact of increased sampling on resolving enigmaticrelationships. Syst Biol 54:317–337.

Ford F (2006) A splitting headache: relationships and generic boundaries among Australian murids. Biol. J. Linn. Soc. 89:117-138.

Ford F (2006) A splitting headache: relationships and generic boundaries among Australian murids. Biol J Linn Soc-Lond 89:117–138.

Fulton TL, Strobeck C (2007) Novel phylogeny of the raccoon family (Procyonidae:Carnivora) based on nuclear and mitochondrial DNA evidence. Mol Phylogenet Evol43:1171–1177.

Gomendio M, Tourmente M, Roldan ERS (2011) Why mammalian lineages respond differently to sexual selection: metabolic rate constrains the evolution of sperm size. Proc. R. Soc. Lond. B 278:3135-3141.

Gómez Montoto L, Magaña C, Tourmente M, Martín-Coello J, Crespo C, Luque-Larena JJ, Gomendio M, Roldan ERS (2011) Sperm competition, sperm numbers and sperm quality in muroid rodents. Plos One 6:e18173.

Holliday JA (2007) Phylogeny and character change in the Feloid Carnivora. PhDDissertation. College of Arts and Sciences, Florida State University.

Johnson WE, Eizirik E, Pecon-Slattery J, Murphy WJ, Antunes A, Teeling E, O'Brien SJ(2006) The late Miocene radiation of modern Felidae: a genetic assessment. Science311:73–77.

Kjer KM, Honeycutt RL (2007) Site specific rates of mitochondrial genomes and thephylogeny of eutheria. BMC Evol Biol 7:8–17.

Krajewski C, Torunsky R, Sipiorski JT, Westerman M (2007) Phylogenetic Relationships of the Dasyurid Marsupial Genus Murexia. J Mammal 88:696-705.

Lecompte E, Aplin K, Denys C, Catzeflis F, Chades M, Chevret P (2008) Phylogeny andbiogeography of African Murinae based on mitochondrial and nuclear gene sequences,with a new tribal classification of the subfamily. BMC Evol Biol 8:199–220.

Liu X, Wei F, Li M, Jiang X, Feng Z, Hu J (2004) Molecular phylogeny and taxonomy ofwood mice (genus Apodemus Kaup, 1829) based on complete mtDNA cytochrome bsequences, with emphasis on Chinese species. Mol Phylogenet Evol 33:1–15.

Marmi J, López-Giráldez JF, Domingo-Roura X (2004) Phylogeny, evolutionary history andtaxonomy of the Mustelidae based on sequences of the cytochrome b gene and a complexrepetitive flanking region. Zool Scr 33:481–499.

May-Collado LJ and Agnarsson I (2006) Cytochrome b and Bayesian inference of whale phylogeny. Mol Phyl Evol 38: 344–354

McGowen MR (2011) Toward the resolution of an explosive radiation—a multilocus phylogeny of oceanic dolphins (Delphinidae). Mol Phyl Evol 60:345-357.

Menzies JI (1996) A systematic revision of Melomys (Rodentia: Muridae) of New Guinea.Aust J Zool 44:367–426.

Meredith RW, Westerman M, Springer MS (2009) A phylogeny of Diprotodontia (Marsupialia) based on sequences for five nuclear genes. Mol Phylogenet Evol 51: 554–571.

Michaux J, Chevret P, Renaud S (2007) Morphological diversity of Old World rats and mice(Rodentia, Muridae) mandible in relation with phylogeny and adaptation. J Zool Syst EvolRes 45:263–279.

Morgan CC (2008) Geometric morphometrics of the scapula of South American caviomorphrodents (Rodentia: Hystricognathi): Form, function and phylogeny. Mammal Biol 74:497–506.

Nikaido M, Matsuno F, Hamilton H, Brownell RLJ, Cao Y, Ding W, Zuoyan Z, Shedlock AM, Fordyce RE, Hasegawa M, Okada N (2001) Retroposon analysis of major cetacean lineages: The monophyly of toothed whales and the paraphyly of river dolphins. PNAS 98:7384-7389.

Nishihara H, Hasegawa M, Okada N (2006) Pegasoferae, an unexpected mammalian cladерevealed by tracking ancient retroposon insertions. PNAS 103:9929–9934.

O'Leary MA, Gatesy J, (2008) Impact of increased character sampling on the phylogeny ofCetartiodactyla (Mammalia): combined analysis including fossils. Cladistics 24:397–442.

Pitra C, Fickel C, Meijaard E, Colin Groves P (2004) Evolution and phylogeny of old worlddeer. Mol Phylogenet Evol 33:880–895.

Poux C, Douzery EJ (2004) Primate phylogeny, evolutionary rate variations, and divergencetimes: a contribution from the nuclear gene IRBP. Am J Phys Anthropol 124:1–16.

Prasad AB, Allard MW, Program NCS, Green ED (2008) Confirming the phylogeny of mammals by use of large comparative sequence data sets. Mol Biol Evol 25:1795-1808.

Prothero DR, Foss SE (2007) The Evolution of Artiodactyls (JHU Press, Baltimore, USA).

Robins JH, Hingston M, Mathisoo-Smith E, Ross HA (2007) Identifying Rattus speciesusing mitochondrial DNA. Mol Ecol Not 7:717–729.

Roos C, Schmitz J, Zischler H (2004) Primate jumping genes elucidate strepsirrhinephylogeny. PNAS 101:10650–10654.

Rowe DL, Honeycutt RL (2002) Phylogenetic relationships, ecological correlates, and molecular evolution within the Cavioidea (Mammalia, Rodentia). Mol. Biol. Evol. 19:263-277.

Rowe KC, Reno ML, Richmond DM, Adkins RM, Steppan SJ (2008) Pliocene colonizationand adaptive radiations in Australia and New Guinea (Sahul): Multilocus systematics ofthe old endemic rodents (Muroidea: Murinae). Mol Phylogenet Evol 47:84–101.

Ruedas LA, Kirsch JA (1997) Systematics of Maxomys Sody, 1936 (Rodentia: Muridae:Murinae): DNALDNA hybridization studies of some Borneo-Javan species and alliedSundaic and Australo-Papuan genera. Biol J Linn Soc-Lond 81:385–408.

Sears KE, Finarelli JA, Flynn JJ, Wyss AR (2008) Estimating body mass in New Worldmonkeys (Platyrrhini, Primates) with a consideration of the Miocene platyrrhine,Chilecebus carrascoensis. American Museum Novitates 3167:1–29.

Seddon JM, Baverstock PR (2000) Evolutionary Lineages of RT1.Ba in the AustralianRattus. Mol Biol Evol 17:768–772.

Springer MS, Murphy WJ (2007) Mammalian evolution and biomedicine: new views fromphylogeny. Biol Rev 82:375–392.

Steiper ME, Ruvolo M (2003) New World monkey phylogeny based on X-linked G6PDDNA sequences. Mol Phylogenet Evol 27:121–130.

Steppan SJ, Adkins RM, Anderson J (2004) Phylogeny and divergence-date estimates ofrapid radiations in Muroid rodents based on multiple nuclear genes. Syst Biol 53:533–553.

Steppan SJ, Adkins RM, Spinks PQ, Hale C (2005) Multigene phylogeny of the Old Worldmice, Murinae, reveals distinct geographic lineages and the declining utility ofmitochondrial genes compared to nuclear genes. Mol Phylogenet Evol 37:370–388.

Swann CA, Cooper SJ, Breed WG (2007) Molecular evolution of the carboxy terminalregion of the zona pellucida 3 glycoprotein in murine rodents. Reproduction 133:697–708.

Valdespino C (2007) Physiological constraints and latitudinal breeding season in the Canidae. Physiol. Biochem. Zool. 80:580-591.

Veron G, Colyn M, Dunham AE, Taylor P, Gaubert P (2004) Molecular systematics andorigin of sociality in mongooses (Herpestidae, Carnivora). Mol Phylogenet Evol 30:582–598.

Watts CH, Baverstock PR (1994) Evolution in New Guinean Muridae (Rodentia) assessedby microcomplement fixation of Albumin. Aust J Zool 42:295–306.

Weisbecker V, Schmid S (2007). Autopodial skeletal diversity in hystricognath rodents:Functional and phylogenetic aspects. Mammal Biol 72:27–44.

Xing J, Wang H, Han K, Ray DA, Huang CH, Chemnick LG, Stewart CB, Disotell TR,Ryder OA, Batzer MA (2005) A mobile element based phylogeny of Old World monkeys. Mol Phylogenet Evol 37:872–880.

Yoder AD, Yang Z (2004) Divergence dates for Malagasy lemurs estimated from multiplegene loci: geological and evolutionary context. Mol Ecol 13:757–773.

Table S1: Data included in study. HW= sperm head width, HL= sperm head length, TSL= total sperm length, BMASS= body mass, TMASS= testes mass, RTM= residual testes mass claculated seperately for each clade.

taxa	Class	Order	Family	Prm1 (root-to-tip ω)	Length Prm1	HW	HL	TSL	HW/ TSL	HL/ TSL	HL/ HW	BMASS (g)	TMASS (g)	log (BMASS)	log (TMASS)	RTM (by clade)	Genbank accession Prm1	References sperm dimensions	References body and testes mass
Antilocapra americana	Eutheria	Artiodactyla	Antilocapridae	0.644	156												EU189418		
Bos taurus	Eutheria	Artiodactyla	Bovidae	0.361	156	4.30	6.77	53.53	0.08	0.13	1.57	680385.00	681.00	5.83	2.83	0.10	NM_174156	Cummins and Woodall 1985; Gage 1998	Kenagy and Trombulak 1986
Capra hircus	Eutheria	Artiodactyla	Bovidae	0.255	156		8.27	59.39		0.14		25420.00	156.80	4.41	2.20	0.05	HM773246	Cummins and Woodall 1985	Anderson et al 2004
Elaphodus cephalophus	Eutheria	Artiodactyla	Cervidae	0.520	156												DQ299383		
Hexaprotodon liberiensis	Eutheria	Artiodactyla	Hippopotamidae	0.311	153												GQ368531		
Hippopotamus amphibius	Eutheria	Artiodactyla	Hippopotamidae	0.311	153	2.90	4.60	33.49	0.09	0.14	1.59	1600000.00	650.00	6.20	2.81	−0.07	EU189424	Cummins and Woodall 1985; Meisner et al 2005	Gage and Freckleton 2003
Moschus sp	Eutheria	Artiodactyla	Moschidae	0.360	156												EU189419		
Ovis aries	Eutheria	Artiodactyla	Bovidae	0.255	156	4.30	8.20	64.70	0.07	0.13	1.91	57172.73	222.99	4.76	2.35	0.05	FJ900270	Cummins and Woodall 1985; Gage 1998	Anderson et al 2004
Ovis dalli	Eutheria	Artiodactyla	Bovidae	0.234	156												EU189417		
Potamochoerus porcus	Eutheria	Artiodactyla	Suidae	0.461	153												EU189425		
Sus scrofa	Eutheria	Artiodactyla	Suidae	0.397	153	5.00	8.50	54.60	0.09	0.16	1.70	39700.00	128.20	4.60	2.11	−0.12	NM_214253	Cummins and Woodall 1985; Gage 1998	Almeida et al 2006
Balaena mysticetus	Eutheria	Cetacea	Balaenidae	0.694	150							51000000.00	162996.00	7.71	5.21	0.44	EU444938		MacLeod 2010
Balaenoptera acutorostrata	Eutheria	Cetacea	Balaenopteridae	1.034	150		5.20	56.70		0.09		12000000.00	8796.00	7.08	3.94	−0.43	EU444935	Plön and Bernard 2006	MacLeod 2010
Balaenoptera bonaerensis	Eutheria	Cetacea	Balaenopteridae	1.034	150												EU444934		
Balaenoptera borealis	Eutheria	Cetacea	Balaenopteridae	0.865	150							18000000.00	16398.00	7.26	4.21	−0.27	EU444932		MacLeod 2010
Balaenoptera edeni	Eutheria	Cetacea	Balaenopteridae	0.865	150	2.13	3.82	56.03	0.04	0.07	1.79	18000000.00	19998.00	7.26	4.30	−0.18	EU444933	Kita et al 2001	MacLeod 2010
Balaenoptera musculus	Eutheria	Cetacea	Balaenopteridae	0.947	150							107000000.00	69978.00	8.03	4.84	−0.13	EU444931		MacLeod 2010
Balaenoptera physalus	Eutheria	Cetacea	Balaenopteridae	0.817	150							51000000.00	58293.00	7.71	4.77	−0.01	EU444930		MacLeod 2010
Berardius bairdii	Eutheria	Cetacea	Ziphidae	1.885	150	1.45	4.28	51.60	0.03	0.08	2.95	11380000.00	10003.02	7.06	4.00	−0.35	GQ368522	Kita et al 2001	MacLeod 2010
Caperea marginata	Eutheria	Cetacea	Neobalaenidae	0.356	150							2900000.00	1899.50	6.46	3.28	−0.69	EU444937		MacLeod 2010
Cephalorhynchus commersonii	Eutheria	Cetacea	Delphinidae	0.402	144							78000.00	1220.00	4.89	3.09	0.12	JF505015		MacLeod 2010
Delphinapterus leucas	Eutheria	Cetacea	Monodontidae	0.323	147		3.80										GQ368517	Miller et al 2002	
Delphinus capensis	Eutheria	Cetacea	Delphinidae	0.366	141	1.90	3.90				2.05						JF505005	Meisner et al 2005	
Delphinus delphis	Eutheria	Cetacea	Delphinidae	0.366	141	2.08	4.29	70.59	0.03	0.06	2.06	121966.79	4010.27	5.09	3.60	0.51	EU697408	Kita et al 2001; Plön and Bernard 2006	MacLeod 2010
Eschrichtius robustus	Eutheria	Cetacea	Eschrichtiidae	0.865	150							25000000.00	67500.00	7.40	4.83	0.26	EU444936		MacLeod 2010
Eubalaena australis	Eutheria	Cetacea	Balaenopteridae	0.476	150												GQ368526		
Eubalaena glacialis	Eutheria	Cetacea	Balaenopteridae	0.476	150							74000000.00	972000.00	7.87	5.99	1.11	GQ368527		Brownell and Ralls 1986
Eubalaena japonica	Eutheria	Cetacea	Balaenopteridae	0.476	150												EU444939		
Feresa attenuata	Eutheria	Cetacea	Delphinidae	0.475	144												GQ368511		
Globicephala macrorhynchus	Eutheria	Cetacea	Delphinidae	0.439	144	2.00	4.55	74.12	0.03	0.06	2.27						GQ368512	Kita et al 2001	
Grampus griseus	Eutheria	Cetacea	Delphinidae	0.439	144	2.00	4.47	74.40	0.03	0.06	2.23	400000.00	7000.00	5.60	3.85	0.42	GQ368513	Kita et al 2001	Freeman 1990
Inia geoffrensis	Eutheria	Cetacea	Iniidae	0.365	147							122000.00	1447.04	5.09	3.16	0.07	GQ368529		MacLeod 2010
Kogia breviceps	Eutheria	Cetacea	Physeteridae	1.252	150		3.40	50.50		0.07							GQ368525	Plön and Bernard 2006	MacLeod 2010
Kogia sima	Eutheria	Cetacea	Physeteridae	1.380	150	1.80	3.50	32.60	0.06	0.11	1.94	350000.00	4000.00	5.54	3.60	0.22	GQ368524	Plön and Bernard 2006; Meisner et al 2005	Freeman 1990
Lagenodelphis hosei	Eutheria	Cetacea	Delphinidae	0.366	141												JF505011		
Lagenorhynchus acutus	Eutheria	Cetacea	Delphinidae	0.403	141	1.25	4.00				3.20	182500.00	298.82	5.26	2.48	−0.73	GQ368510	Neuenhagen et al 2007	Neuenhagen et al 2007
Lagenorhynchus albirostris	Eutheria	Cetacea	Delphinidae	0.402	144												JF505014		
Lagenorhynchus obliquidens	Eutheria	Cetacea	Delphinidae	0.332	141	1.96	4.23	69.26	0.03	0.06	2.16	90000.00	1100.00	4.95	3.04	0.04	JF505013	Kita et al 2001; Miller et al 2002	Freeman 1990
Lipotes vexillifer	Eutheria	Cetacea	Lipotidae	0.405	147												JF701667		

Lissodelphis borealis	Eutheria	Cetacea	Delphinidae	0.333	144												GQ368509		
Megaptera novaeangliae	Eutheria	Cetacea	Balaenopteridae	0.817	150		4.00	52.50		0.08		40000000.00	38000.00	7.60	4.58	−0.13	EU444929	Plön and Bernard 2006	MacLeod 2010
Mesoplodon bidens	Eutheria	Cetacea	Ziphidae	1.516	150												GQ368520		
Mesoplodon grayi	Eutheria	Cetacea	Ziphidae	1.884	150												GQ368519		
Mesoplodon peruvianus	Eutheria	Cetacea	Ziphidae	1.699	150												GQ368518		
Monodon monoceros	Eutheria	Cetacea	Monodontidae	0.327	147												GQ368516		
Neophocaena phocaenoides	Eutheria	Cetacea	Phocaenidae	0.502	144	2.11	3.58	62.72	0.03	0.06	1.70	71800.00	1772.02	4.86	3.25	0.31	GQ368515	Kita et al 2001; Li et al 2009	MacLeod 2010
Orcaella heinsohni	Eutheria	Cetacea	Delphinidae	0.366	144												JF505017		
Orcinus orca	Eutheria	Cetacea	Delphinidae	0.439	144	3.88	4.43	74.44	0.05	0.06	1.14	7365000.00	10311.00	6.87	4.01	−0.22	EU697405	Kita et al 2001; Miller et al 2002	MacLeod 2010
Peponocephala electra	Eutheria	Cetacea	Delphinidae	0.475	144							206000.00	1795.08	5.31	3.25	0.02	JF505016		MacLeod 2010
Phocoena phocoena	Eutheria	Cetacea	Phocaenidae	0.502	144		5.85	73.80		0.08		58000.00	2320.00	4.76	3.37	0.48	EU697404	Plön and Bernard 2006	Westgate and Read 2007; MacLeod 2010
Phocoenoides dalli	Eutheria	Cetacea	Phocaenidae	0.502	144	2.16	3.98	60.48	0.04	0.07	1.84	145200.00	382.02	5.16	2.58	−0.56	GQ368514	Kita et al 2001	MacLeod 2010
Physeter catodon	Eutheria	Cetacea	Physeteridae	3.579	150												EU444927		
Platanista minor	Eutheria	Cetacea	Platanistidae	0.625	150												GQ368523		
Pontoporia blainvillei	Eutheria	Cetacea	Pontoporiidae	0.470	147												EU189421		
Pseudorca crassidens	Eutheria	Cetacea	Delphinidae	0.439	144												EU697406		
Sotalia fluviatilis	Eutheria	Cetacea	Delphinidae	0.366	141												JF505012		
Sousa chinensis	Eutheria	Cetacea	Delphinidae	0.366	144												JF505010		
Stenella attenuata	Eutheria	Cetacea	Delphinidae	0.366	141							80500.00	1029.03	4.91	3.01	0.04	JF505009		MacLeod 2010
Stenella coeruleoalba	Eutheria	Cetacea	Delphinidae	0.403	144												EU697409		
Stenella frontalis	Eutheria	Cetacea	Delphinidae	0.366	141												JF505008		
Stenella longirostris	Eutheria	Cetacea	Delphinidae	0.366	141												JF505007		
Steno bredanensis	Eutheria	Cetacea	Delphinidae	0.366	144	2.00	3.60				1.80						EU697407	Meisner et al 2005	
Tasmacetus shepherdi	Eutheria	Cetacea	Ziphidae	1.516	150												GQ368521		
Tursiops aduncus	Eutheria	Cetacea	Delphinidae	0.403	144												JF505006		
Tursiops truncatus	Eutheria	Cetacea	Delphinidae	0.403	144	2.13	4.41	70.16	0.03	0.06	2.07	146250.00	756.40	5.17	2.88	−0.26	GQ368508	Kita et al 2001; Plön and Bernard 2006	MacLeod 2010
Ziphius cavirostris	Eutheria	Cetacea	Delphinidae	1.887	150							3000000.00	8001.00	6.48	3.90	−0.08	EU444928		MacLeod 2010
Chalinolobus beatrix	Eutheria	Chiroptera	Vespertilionidae	0.305	141												AF435944		
Chilonatalus micropus	Eutheria	Chiroptera	Natalidae	0.121	153												AF435936		
Corynorhinus townsendii	Eutheria	Chiroptera	Vespertilionidae	0.207	147												AF435940		
Desmodus rotundus	Eutheria	Chiroptera	Phyllostomidae	0.046	147	2.71	4.71				1.74	33.70	0.08	1.53	−1.08	−0.11	AF435934	Forman 1979 (?)	Wilkinson and McCracken 2003
Eptesicus brasiliensis	Eutheria	Chiroptera	Vespertilionidae	0.162	147												AF435943		
Eptesicus fuscus	Eutheria	Chiroptera	Vespertilionidae	0.162	147	2.00	5.00	72.00	0.03	0.07	2.50	15.50		1.19			AF435942	Cummins and Woodall 2003	Hosken 1997
Galeopterus variegatus	Eutheria	Chiroptera	Chiroptera	0.476	144												AF435927		
Hipposideros commersoni	Eutheria	Chiroptera	Hipposideridae	0.478	150												AF435929		
Hypsugo savii	Eutheria	Chiroptera	Vespertilionidae	0.152	141												AF435945		
Monophyllus redmani	Eutheria	Chiroptera	Phyllostomidae	0.036	147												AF435935		
Mormoops megalophylla	Eutheria	Chiroptera	Mormoopidae	0.089	147												AF435933		
Murina cyclotis	Eutheria	Chiroptera	Vespertilionidae	0.140	147												AF435947		
Myotis daubentonii	Eutheria	Chiroptera	Vespertilionidae	0.184	144												AF435946		
Myotis lucifugus	Eutheria	Chiroptera	Vespertilionidae	0.201	144	1.80	4.30	51.00	0.04	0.08	2.39	6.80	0.11	0.83	−0.97	0.05	BK006489	Cummins and Woodall 2008	Hosken 1997
Natalus stramineus	Eutheria	Chiroptera	Natalidae	0.145	153												AF435937		
Neoromicia brunneus	Eutheria	Chiroptera	Vespertilionidae	0.152	141												AF435941		
Otonycteris hemprichii	Eutheria	Chiroptera	Vespertilionidae	0.273	153												AF435938		
Plecotus auritus	Eutheria	Chiroptera	Vespertilionidae	0.212	153							7.20	0.04	0.86	−1.36	−0.34	AF435939		Wilkinson and McCraken 2003
Pteronotus parnellii	Eutheria	Chiroptera	Mormoopidae	0.038	147												AF435932		
Pteropus hypomelanus	Eutheria	Chiroptera	Pteropodidae	0.000	150												AF435928		

Rhinolophus ferrumequinum	Eutheria	Chiroptera	Rhinolophidae	0.668	159		6.40					14.00		1.15			AF435930	Gage 1998	Gage 1998
Rhinopoma hardwickii	Eutheria	Chiroptera	Rhinopomatidae	0.243	150							11.00	0.25	1.04	−0.60	0.40	AF435931		Wilkinson and McCraken 2003
Ateles paniscus	Eutheria	Primates	Atelidae	0.359	153	3.14	6.40	65.10	0.05	0.10	2.04	9800.00	64.23	3.99	1.81	0.49	AF119242	Anderson et al 2005; González-Moreno et al 2000	Dixson and Anderson 2004
Colobus guereza	Eutheria	Primates	Cercopithecidae	0.714	156												AF119233		
Gorilla gorilla	Eutheria	Primates	Pongidae	0.536	156		4.78	61.17		0.08		134000.00	23.20	5.13	1.37	−0.64	AF215709	Cummins and Woodall 1985	Kenagy and Trombulak 1986
Homo sapiens	Eutheria	Primates	Hominidae	0.595	156		4.50	56.90		0.08		63540.00	50.20	4.80	1.70	−0.11	Y00443	Anderson et al 2005	Kenagy and Trombulak 1986
Hylobates lar	Eutheria	Primates	Hylobatidae	0.479	156		7.20	63.30		0.11		5500.00	5.50	3.74	0.74	−0.43	L14588	Anderson et al 2005	Kenagy and Trombulak 1986
Macaca fascicularis	Eutheria	Primates	Cercopithecidae	0.447	156	3.50	5.53	73.63	0.05	0.08	1.58	4787.00	35.70	3.68	1.55	0.42	AB169169	Cummins and Woodall 1985	Kenagy and Trombulak 1986
Macaca mulatta	Eutheria	Primates	Cercopithecidae	0.395	156	3.20	5.30	74.10	0.04	0.07	1.66	10430.00	76.00	4.02	1.88	0.54	NM_001143822	Anderson et al 2005; Cummins and Woodall 1985	Kenagy and Trombulak 1986
Nasalis larvatus	Eutheria	Primates	Cercopithecidae	0.716	156							19833.33	11.90	4.30	1.08	−0.43	AF119237		Kramer and Kuehl 1980
Otolemur garnettii	Eutheria	Primates	Lemuridae	0.000	150												BK006490		
Pan paniscus	Eutheria	Primates	Pongidae	0.531	153		4.70	68.10		0.07		39100.00	135.20	4.59	2.13	0.45	L14590	Anderson et al 2005	Dixson and Anderson 2004
Pan troglodytes	Eutheria	Primates	Pongidae	0.629	156		4.68	57.36		0.08		44340.00	118.80	4.65	2.07	0.36	AF215708	Cummins and Woodall 1985	Kenagy and Trombulak 1986
Papio cynocephalus	Eutheria	Primates	Cercopithecidae	0.395	156		4.60	76.00		0.06		28610.00	78.60	4.46	1.90	0.29	AF119239	Anderson et al 2005	Gage and Freckleton 2003
Piliocolobus badius	Eutheria	Primates	Cercopithecidae	0.554	156												AF294850		
Pongo pygmaeus	Eutheria	Primates	Pongidae	0.490	156		5.31	66.58		0.08		74640.00	35.30	4.87	1.55	−0.31	AF215710	Cummins and Woodall 1985	Kenagy and Trombulak 1986
Saguinus imperator	Eutheria	Primates	Callithricidae	0.628	153												X61678		
Saimiri sciureus	Eutheria	Primates	Cebidae	0.481	153		5.11	69.24		0.07		779.00	3.17	2.89	0.50	−0.16	AF119241	Cummins and Woodall 1985	Kenagy and Trombulak 1986
Semnopithecus entellus	Eutheria	Primates	Cercopithecidae	0.426	153							18500.00	11.10	4.27	1.05	−0.44	AF119235		Harrison and Lewis 1986
Trachypithecus cristatus	Eutheria	Primates	Cercopithecidae	0.532	156												AF294861		
Trachypithecus francoisi	Eutheria	Primates	Cercopithecidae	0.553	156												AF119234		
Trachypithecus geei	Eutheria	Primates	Cercopithecidae	0.553	156												AF294857		
Trachypithecus johnii	Eutheria	Primates	Cercopithecidae	0.357	156												AF294853		
Trachypithecus obscurus	Eutheria	Primates	Cercopithecidae	0.532	156												AF119238		
Trachypithecus phayrei crepuscula	Eutheria	Primates	Cercopithecidae	0.532	153												AF294858		
Trachypithecus pileatus	Eutheria	Primates	Cercopithecidae	0.486	156												AF294856		
Trachypithecus vetulus	Eutheria	Primates	Cercopithecidae	0.426	153												AF119236		
Acomys cahirinus	Eutheria	Rodentia	Muridae	0.090	153	2.60	7.70	124.00	0.02	0.06	2.96	75.50	0.51	1.88	−0.29	−0.21	Ramm et al. 2008	Gage 1998	Ramm et al 2005
Acomys cilcicus	Eutheria	Rodentia	Muridae	0.090	153							52.68	0.47	1.72	−0.32	−0.10	Ramm et al. 2008		Ramm et al 2008
Apodemus sylvaticus	Eutheria	Rodentia	Muridae	0.060	159	5.32	9.24	126.15	0.04	0.07	1.74	30.43	0.96	1.48	−0.02	0.44	FJ411394	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Arvicola sapidus	Eutheria	Rodentia	Cricetidae	0.086	153	4.02	7.09	115.54	0.03	0.06	1.76	217.67	2.24	2.34	0.35	−0.01	Lüke et al. 2011	Gómez Montoto et al 2011b	L. Gómez-Montoto, E.R.S. Roldan and M. Gomendio, unpublished data
Arvicola terrestris	Eutheria	Rodentia	Cricetidae	0.101	153			118.32				91.56	0.41	1.96	−0.39	−0.39	Lüke et al. 2011	Lüke et al. 2014a	Gómez Montoto et al 2011
Cavia porcellus	Eutheria	Rodentia	Caviidae	0.379	147	6.60	10.87	114.07	0.06	0.10	1.65	813.30	4.10	2.91	0.61	−0.29	NM_001173006	Cummins and Woodall 1985; Gallardo et al 2002	Kenagy and Trombulak 1986
Chionomys nivalis	Eutheria	Rodentia	Cricetidae	0.150	153	4.67	8.40	105.20	0.04	0.08	1.80	49.25	0.99	1.69	−0.01	0.25	Lüke et al. 2011	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Clethrionomys glareolus	Eutheria	Rodentia	Cricetidae	0.102	150	3.70	6.73	83.40	0.04	0.08	1.82	25.25	0.43	1.40	−0.37	0.16	Lüke et al. 2011	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Cricetulus griseus	Eutheria	Rodentia	Cricetidae	0.041	156	2.41	13.47	249.28	0.01	0.05	5.59	33.72	1.78	1.53	0.25	0.66	Lüke et al. 2011	Cummins and Woodall 1985	Lüke et al. 2011
Meriones unguiculatus	Eutheria	Rodentia	Muridae	0.039	153	2.90	4.50	138.50	0.02	0.03	1.55	76.80	1.08	1.89	0.04	0.11	Ramm et al. 2008	Marston and Chang 1966	Blottner et al 2000
Mesocricetus auratus	Eutheria	Rodentia	Cricetidae	0.023	156	3.01	9.00	189.22	0.02	0.05	2.99	124.99	3.50	2.10	0.54	0.41	Lüke et al. 2011	Gage and Freckleton 2003	Lüke et al. 2011
Micromys minutus	Eutheria	Rodentia	Muridae	0.041	156		5.70	63.70		0.09		7.80	0.18	0.89	−0.74	0.27	Ramm et al. 2008	Cummins and Woodall 1985	Kenagy and Trombulak 1986
Microtus agrestis	Eutheria	Rodentia	Cricetidae	0.121	153		6.90	103.50		0.07		46.60	0.80	1.67	−0.10	0.18	Lüke et al. 2011	Cummins and Woodall 1985	Kenagy and Trombulak 1986
Microtus arvalis	Eutheria	Rodentia	Cricetidae	0.171	153	3.36	6.85	91.70	0.04	0.07	2.04	45.90	0.48	1.66	−0.32	−0.03	Lüke et al. 2011	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Microtus cabrerae	Eutheria	Rodentia	Cricetidae	0.221	153	4.21	6.46	85.70	0.05	0.08	1.53	46.30	0.15	1.67	−0.81	−0.53	Lüke et al. 2011	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a

Microtus gerbei	Eutheria	Rodentia	Cricetidae	0.121	153												Lüke et al. 2011		
Mus cookii	Eutheria	Rodentia	Muridae	0.075	156	3.60	7.20	105.20	0.03	0.07	2.00	23.67	0.30	1.37	−0.52	0.04	FJ411374		Gómez Montoto et al 2011
Mus famulus	Eutheria	Rodentia	Muridae	0.089	156	3.40	6.70	102.50	0.03	0.07	1.97	27.40	0.05	1.44	−1.28	−0.78	FJ411378		Gómez Montoto et al 2011
Mus macedonicus	Eutheria	Rodentia	Muridae	0.075	156	3.40	7.00	100.08	0.03	0.07	2.06	20.10	0.30	1.30	−0.53	0.10	FJ411380		Gómez Montoto et al 2011
Mus musculus bactrianus	Eutheria	Rodentia	Muridae	0.075	156	3.20	7.10	112.90	0.03	0.06	2.22	18.06	0.17	1.26	−0.76	−0.09	FJ411373		Gómez Montoto et al 2011
Mus musculus castaneus	Eutheria	Rodentia	Muridae	0.075	156	3.40	7.70	122.70	0.03	0.06	2.26	18.82	0.08	1.27	−1.12	−0.47	FJ411375		Gómez Montoto et al 2011
Mus musculus domesticus	Eutheria	Rodentia	Muridae	0.072	156	3.90	7.90	123.75	0.03	0.06	2.03	22.05	0.11	1.34	−0.96	−0.38	FJ411377		Gómez Montoto et al 2011
Mus musculus musculus	Eutheria	Rodentia	Muridae	0.075	156	3.67	9.25	118.39	0.03	0.08	2.52	21.85	0.14	1.34	−0.86	−0.27	FJ411376	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Mus pahari	Eutheria	Rodentia	Muridae	0.089	156	5.01	9.71	135.85	0.04	0.07	1.94	33.15	0.13	1.52	−0.89	−0.48	FJ411379	Gómez-Montoto L, Roldan ERS and Gomendio M, unpublished data	Gómez Montoto et al 2011a
Mus spicilegus	Eutheria	Rodentia	Muridae	0.072	156	3.40	8.41	99.83	0.03	0.08	2.47	18.10	0.43	1.26	−0.37	0.30	FJ411381	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Mus spretus	Eutheria	Rodentia	Muridae	0.075	156	3.72	8.00	105.83	0.04	0.08	2.15	18.17	0.30	1.26	−0.52	0.14	FJ411382	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Peromyscus californicus	Eutheria	Rodentia	Cricetidae	0.021	153		5.30	72.00		0.07		34.20	0.20	1.53	−0.70	−0.29	Ramm et al. 2008	Cummins and Woodall 1985	Lemaitre et al 2009
Peromyscus leucopus	Eutheria	Rodentia	Cricetidae	0.021	153		5.30	74.80		0.07		24.20	0.38	1.38	−0.42	0.13	Ramm et al. 2008	Cummins and Woodall 1985	Lemaitre et al 2009
Peromyscus maniculatus	Eutheria	Rodentia	Cricetidae	0.021	153		5.20	75.00		0.07		19.00	0.27	1.28	−0.58	0.07	Ramm et al. 2008	Cummins and Woodall 1985	Trainor et al 2006
Peromyscus polionotus	Eutheria	Rodentia	Cricetidae	0.020	153	3.10	5.40	74.80	0.04	0.07	1.74	14.50	0.18	1.16	−0.74	0.02	Ramm et al. 2008	Cummins and Woodall 1985	Trainor et al 2006
Phodopus campbelli	Eutheria	Rodentia	Cricetidae	0.076	153	2.99	7.37	118.06	0.03	0.06	2.46	48.52	1.93	1.69	0.29	0.55	Lüke et al. 2011	Lüke et al. 2014a	Lüke et al. 2011
Phodopus roborovski	Eutheria	Rodentia	Cricetidae	0.039	153	3.94	8.71	146.43	0.03	0.06	2.21	25.72	1.06	1.41	0.03	0.55	Lüke et al. 2011	Lüke et al. 2014a	Lüke et al. 2011
Phodopus sungorus	Eutheria	Rodentia	Cricetidae	0.046	153	3.30	8.20	132.23	0.02	0.06	2.48	45.92	1.04	1.66	0.02	0.30	Lüke et al. 2011	Gage and Freckleton 2003; Gage 1998	Lüke et al. 2011
Pitimys duodecimcostatus	Eutheria	Rodentia	Cricetidae	0.140	153	3.00	4.75	58.87	0.05	0.08	1.59	27.52	0.08	1.44	−1.08	−0.59	Lüke et al. 2011	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Pitimys lusitanicus	Eutheria	Rodentia	Cricetidae	0.121	153	3.15	5.78	86.02	0.04	0.07	1.84	18.59	0.12	1.27	−0.94	−0.28	Lüke et al. 2011	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Rattus norvegicus	Eutheria	Rodentia	Muridae	0.057	156		11.90	189.40		0.06		379.63	3.06	2.58	0.49	−0.10	BC126070	Cummins and Woodall 1985	Kenagy and Trombulak 1986; Slott et al 1990; Kawaguchi et al 2004
Sigmodon hispidus	Eutheria	Rodentia	Cricetidae	0.043	156	3.20	6.10	87.00	0.04	0.07	1.91	223.00	1.73	2.35	0.24	−0.13	EU980395	Cummins and Woodall 1985	Kenagy and Trombulak 1986
Antechinomys laniger	Metatheria	Dasyuromorpha	Dasyuridae	0.000	189												AF001587		
Antechinus bellus	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192												AF038295		
Antechinus flavipes	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							48.80	0.34	1.69	−0.47	0.05	AF038293		Taggart et al 1998
Antechinus godmani	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							105.00	0.20	2.02	−0.71	−0.42	AF038296		Taggart et al 1998
Antechinus leo	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192												AF038297		
Antechinus minimus	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							51.00	0.46	1.71	−0.34	0.16	AF038294		Taggart et al 1998
Antechinus naso	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192												AF038301		
Antechinus stuartii	Metatheria	Dasyuromorpha	Dasyuridae	0.000	195		11.40	271.10		0.04		40.00	0.64	1.60	−0.19	0.38	L35335	Cummins and Woodall 1985	Taggart et al 1998
Antechinus swainsonii	Metatheria	Dasyuromorpha	Dasyuridae	1.162	189							71.40	0.63	1.85	−0.20	0.20	L35338		Taggart et al 1998
Dasycercus cristicauda	Metatheria	Dasyuromorpha	Dasyuridae	0.371	192	1.90	9.50				5.00	74.50	0.77	1.87	−0.11	0.27	AF010270	Cummins and Woodall 1985	Taggart et al 1998
Dasykulata rosamondae	Metatheria	Dasyuromorpha	Dasyuridae	0.186	192	1.60	9.80				6.12	35.30	0.13	1.55	−0.90	−0.29	L35325	Cummins and Woodall 1985	Taggart et al 1998
Dasyuroides byrnei	Metatheria	Dasyuromorpha	Dasyuridae	0.944	192	2.50	12.70	254.80	0.01	0.05	5.08	123.60	1.38	2.09	0.14	0.38	AF010271	Cummins and Woodall 1985	Taggart et al 1998
Dasyurus albopunctatus	Metatheria	Dasyuromorpha	Dasyuridae	0.314	189												AF010272		
Dasyurus geoffroii	Metatheria	Dasyuromorpha	Dasyuridae	0.314	189												AF010274		
Dasyurus hallucatus	Metatheria	Dasyuromorpha	Dasyuridae	0.301	186							796.00	2.49	2.90	0.40	0.08	L35341		Taggart et al 1998
Dasyurus maculatus	Metatheria	Dasyuromorpha	Dasyuridae	0.301	189												AF010276		
Dasyurus spartacus	Metatheria	Dasyuromorpha	Dasyuridae	0.314	189												AF010275		
Dasyurus viverrinus	Metatheria	Dasyuromorpha	Dasyuridae	0.301	186	1.90	11.00				5.79	1300.00	3.00	3.11	0.48	0.01	L35340	Cummins and Woodall 1985	Taggart et al 1998
Micromurexia habbema	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							35.00	0.23	1.54	−0.64	−0.02	AF038300		Taggart et al 1998
Murexchinus melanurus	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							45.20	0.25	1.66	−0.60	−0.07	AF038299		Taggart et al 1998
Murexia longicaudata	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							78.20	0.37	1.89	−0.43	−0.06	L35336		Taggart et al 1998

Myoictis melas	Metatheria	Dasyuromorpha	Dasyuridae	0.186	192							117.50	0.73	2.07	−0.13	0.12	AF010268		Taggart et al 1998
Myoictis wallacei	Metatheria	Dasyuromorpha	Dasyuridae	0.186	192							240.80	0.87	2.38	−0.06	−0.03	AF010269		Taggart et al 1998
Myrmecobius fasciatus	Metatheria	Dasyuromorpha	Myrmecobiidae	0.000	192		10.90	138.40		0.08							U87139	Gage 1998	
Neophascogale lorentzii	Metatheria	Dasyuromorpha	Dasyuridae	0.220	189							237.00	0.71	2.37	−0.15	−0.10	AF010267		Taggart et al 1998
Ningaui ridei	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							9.50	0.09	0.98	−1.06	−0.06	AF001588		Taggart et al 1998
Ningaui timealeyi	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							9.40	0.05	0.97	−1.33	−0.32	AF001590		Taggart et al 1998
Ningaui yvonnae	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							10.00	0.07	1.00	−1.13	−0.14	AF001589		Taggart et al 1998
Paramurexia rothschildi	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192												AF038302		
Parantechinus apicalis	Metatheria	Dasyuromorpha	Dasyuridae	0.186	192							63.60	0.48	1.80	−0.32	0.12	L35326		Taggart el al 2003
Phascogale calura	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192												AF038303		
Phascogale tapoatafa	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192												L35327		
Phascolosorex dorsalis	Metatheria	Dasyuromorpha	Dasyuridae	0.220	189							52.60	0.35	1.72	−0.46	0.03	L35339		Taggart et al 1998
Planigale gilesi	Metatheria	Dasyuromorpha	Dasyuridae	1.148	189												AF001593		
Planigale maculata maculata	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192												AF001591		
Planigale maculata sinualis	Metatheria	Dasyuromorpha	Dasyuridae	0.578	189												AF001592		
Planigale tenuirostris	Metatheria	Dasyuromorpha	Dasyuridae	1.788	192												AF001594		
Pseudantechinus bilarni	Metatheria	Dasyuromorpha	Dasyuridae	0.186	189							22.50	0.06	1.35	−1.21	−0.47	AF010277		Taggart el al 2003
Pseudantechinus macdonnellensis	Metatheria	Dasyuromorpha	Dasyuridae	0.186	192							26.00	0.29	1.41	−0.54	0.16	L35337		Taggart el al 2003
Pseudantechinus ningbing	Metatheria	Dasyuromorpha	Dasyuridae	0.186	192							23.00	0.09	1.36	−1.03	−0.29	AF010278		Taggart el al 2003
Pseudantechinus woolleyae	Metatheria	Dasyuromorpha	Dasyuridae	0.186	192												AF010279		
Sarcophilus harrisi	Metatheria	Dasyuromorpha	Dasyuridae	0.301	189	2.20	11.10	218.40	0.01	0.05	5.05	9000.00	6.35	3.95	0.80	−0.24	L35324	Cummins and Woodall 1985	Taggart et al 1998; Keeley et al 2012
Sminthopsis aitkeni	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							18.00	0.11	1.26	−0.96	−0.15	AF089871		Taggart et al 1998
Sminthopsis archeri	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192												AF089872		
Sminthopsis bindi	Metatheria	Dasyuromorpha	Dasyuridae	0.181	192							15.30	0.11	1.18	−0.97	−0.11	AF089873		Taggart et al 1998
Sminthopsis crassicaudata	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192		12.80	264.90		0.05		14.60	0.17	1.16	−0.78	0.09	L32743	Gage 1998	Taggart et al 1998
Sminthopsis dolichura	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							16.00	0.14	1.20	−0.85	0.00	AF089874		Taggart et al 1998
Sminthopsis douglasi	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192												AF089875		
Sminthopsis gilberti	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192												AF089876		
Sminthopsis granulipes	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							18.50	0.28	1.27	−0.55	0.25	AF089877		Taggart et al 1998
Sminthopsis griseoventer	Metatheria	Dasyuromorpha	Dasyuridae	0.372	192							20.00	0.15	1.30	−0.82	−0.04	AF089878		Taggart et al 1998
Sminthopsis hirtipes	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							16.50	0.15	1.22	−0.81	0.03	AF089879		Taggart et al 1998
Sminthopsis leucopus	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							22.50	0.23	1.35	−0.63	0.11	AF089880		Taggart et al 1998
Sminthopsis longicaudata	Metatheria	Dasyuromorpha	Dasyuridae	0.091	189												AF089881		
Sminthopsis macroura	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							20.00	0.24	1.30	−0.62	0.16	AF001586		Taggart et al 1998
Sminthopsis murina	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							20.50	0.27	1.31	−0.58	0.20	AF001585		Taggart et al 1998
Sminthopsis ooldea	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							12.00	0.11	1.08	−0.97	−0.04	AF089882		Taggart et al 1998
Sminthopsis psammophila	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192												AF089883		
Sminthopsis virginiae	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							31.00	0.25	1.49	−0.60	0.05	AF089884		Taggart et al 1998
Sminthopsis youngsoni	Metatheria	Dasyuromorpha	Dasyuridae	0.000	192							11.00	0.12	1.04	−0.94	0.02	AF089885		
Thylacinus cynocephalus	Metatheria	Dasyuromorpha	Thylacinidae	0.000	192												U87140		
Aepyprymnus rufescens	Metatheria	Diprotodontia	Potoroidae	0.246	186	2.60	5.90	106.40	0.02	0.06	2.27	2400.00	4.68	3.38	0.67	−0.03	AF187547	Taggart et al 1995	Taggart et al 1998
Bettongia penicillata	Metatheria	Diprotodontia	Potoroidae	0.288	189	2.30	7.50	162.50	0.01	0.05	3.26	872.00	1.67	2.94	0.22	−0.15	AF187546	Taggart et al 1995	Taggart et al 1998

Dendrolagus dorianus	Metatheria	Diprotodontia	Macropodidae	0.364	189												AF187536		
Dendrolagus goodfellowi	Metatheria	Diprotodontia	Macropodidae	0.244	186												AF187537		
Dorcopsis veterum	Metatheria	Diprotodontia	Macropodidae	0.364	189												AF187540		
Dorcopsulus vanheurni	Metatheria	Diprotodontia	Macropodidae	0.418	189												AF187539		
Hypsiprymnodon moschatus	Metatheria	Diprotodontia	Hypsiprimmodontidae	0.147	195	1.31	8.50	89.25	0.01	0.10	6.49						AF187545	Lloyd et al 2002	
Lagorchestes hirsutus	Metatheria	Diprotodontia	Macropodidae	0.378	198	3.30	6.00	88.40	0.04	0.07	1.82						AF187544	Johnston et al 2003	
Lagostrophus fasciatus	Metatheria	Diprotodontia	Macropodidae	0.166	189												AY189936		
Macropus agilis	Metatheria	Diprotodontia	Macropodidae	0.331	186	3.20	7.50	113.20	0.03	0.07	2.34	11400.00	25.64	4.06	1.41	0.20	L35451	Cummins and Woodall 1985	Taggart et al 1998
Macropus eugenii	Metatheria	Diprotodontia	Macropodidae	0.188	189	3.38	6.67	106.40	0.03	0.06	1.97	7050.00	32.90	3.85	1.52	0.46	L35450	Cummins and Woodall 1985	Paris et al 2005
Macropus giganteus	Metatheria	Diprotodontia	Macropodidae	0.179	186	2.20	7.30	118.90	0.02	0.06	3.32	40720.00	42.02	4.61	1.62	0.00	L35333	Cummins and Woodall 1985	Taggart et al 1998
Macropus parryi	Metatheria	Diprotodontia	Macropodidae	0.390	186	4.89	9.27	119.53	0.04	0.08	1.90						AF187533	Cummins and Woodall 1985	
Macropus rufogriseus	Metatheria	Diprotodontia	Macropodidae	0.207	189							18500.00	54.57	4.27	1.74	0.37	L35329		Rose et al 1997
Macropus rufus	Metatheria	Diprotodontia	Macropodidae	0.176	183	3.40	5.10	123.90	0.03	0.04	1.50	39825.00	38.18	4.60	1.58	−0.04	L35447	Cummins and Woodall 1985	Taggart et al 1998
Onychogalea fraenata	Metatheria	Diprotodontia	Macropodidae	0.259	186												AF187542		
Onychogalea unguifera	Metatheria	Diprotodontia	Macropodidae	0.259	186												AF187543		
Petrogale concinna	Metatheria	Diprotodontia	Macropodidae	0.328	183												AF187538		
Petrogale xanthopus	Metatheria	Diprotodontia	Macropodidae	0.364	189	2.30	7.70	101.20	0.02	0.08	3.35						AF187535	Taggart et al 1995	
Phascolarctos cinereus	Metatheria	Diprotodontia	Phascolarctidae	0.496	183	3.32	11.75	83.01	0.04	0.14	3.54	8150.00	3.72	3.91	0.57	−0.53	U87789	Cummins and Woodall 1985	Taggart et al 1998
Potorous longipes	Metatheria	Diprotodontia	Potoroidae	0.146	186												AF187548		
Pseudochirops cupreus	Metatheria	Diprotodontia	Pseudocheiridae	0.236	210	2.60	5.40	90.10	0.03	0.06	2.08						L35334	Cummins and Woodall 1985	
Setonix brachyurus	Metatheria	Diprotodontia	Macropodidae	0.280	186												AF187541		
Thylogale stigmatica	Metatheria	Diprotodontia	Macropodidae	0.471	189	2.20	7.20	110.30	0.02	0.07	3.27						AF187534	Cummins and Woodall 1985	
Trichosurus vulpecula	Metatheria	Diprotodontia	Phalangeridae	0.127	189	2.58	5.53	94.17	0.03	0.06	2.14	3350.00	8.26	3.53	0.92	0.11	L32744	Cummins and Woodall 1985	Taggart et al 1998
Wallabia bicolor	Metatheria	Diprotodontia	Macropodidae	0.197	189	3.05	7.80	109.50	0.03	0.07	2.56	31500.00	14.75	4.50	1.17	−0.38	L35328	Cummins and Woodall 1985	Taggart et al 1998

References for suppl. Table S1

Almeida FF, Leal MC, França LR (2006) Testis morphometry, duration of spermatogenesis, and spermatogenic efficiency in the wild boar (Sus scrofa scrofa). Biol Reprod 75:792–799.20.

Blottner S, Schön J, Jewgenow K (2006) Seasonally activated spermatogenesis is correlated with increased testicular production of testosterone and epidermal growth factor in mink (Mustela vison). Theriogenology 66:1593–1598.

Brownell RL, Ralls K (1986) Potential for sperm competition in baleen whales. Reports of the International Whaling Commission 8(Special Issue):97-112.

Cummins JM, Woodall PF (1985) On mammalian sperm dimensions. J Reprod Fertil 75:153–175.

Dixson AF, Anderson MJ (2004) Sexual behavior, reproductive physiology and sperm competition in male mammals. Physiol Behav 83:361–371.

Forman GL, Genoways HH (1979) Sperm morphology. Pp. 177-204 in R. J. Baker, J. Knox Jones, and D. C. Carter, eds. Biology of bats of the new world family Phyllostomatidae, Part III. Texas Tech University, Lubbock, TX, UK.

Freeman S (1990) The evolution of the scrotum: A new hypothesis. J Theor Biol 145:429-445.

Gage MJG (1998) Mammalian sperm morphometry. Proc Roy Soc Lond B Bio 265:97–103.

Gage MJG, Freckleton R (2003) Relative testis size and sperm morphometry across mammals: no evidence for an association between sperm competition and sperm length. Proc Roy Soc Lond B 270:625–632.

Gallardo MH, Mondaca FC, Ojeda RA, Köhler N, Garrido O (2002) Morphological diversity in the sperms of Caviomorph rodents. J Neotrop Mammal 9:159–170.

Gómez-Montoto, E.R.S. Roldan & M. Gomendio, unpublished data

Gómez Montoto L, Magaña C, Tourmente M, Martín-Coello J, Crespo C et al. (2011) Sperm competition, sperm numbers and sperm quality in Muroid rodents. PLoS ONE 6: e18173.

Gómez Montoto L, Varea Sánchez M, Tourmente M, Martín-Coello J, Luque-Larena JJ, Gomendio M, Roldan ERS (2011b) Sperm competition differentially affects swimming velocity and size of spermatozoa from closely related Muroid rodents—Head first. Reproduction 142:819–830.

González-Moreno MC, Cetica PD, Merani MS (2000) J Primatol 30:309–314.

Harrison RM, Lewis RW (1986) The male reproductive tract and its fluids; in Dukelow WR, Erwin J (eds): Comparative Primate Biology. New York, Liss, vol 3, pp 101–148.

Hosken DJ (1997) Spermcompetition in bats. Proc. R. Soc. Lond. B 264:385–392.

Johnston SD, Ward D, Lemon J, Gunn I, MacCallum CA, Keeley T, Blyde D (2007) Studies of male reproduction in captive African wild dogs (Lycaon pictus). Anim Reprod Sci 100:338–355.

Kawaguchi T, Kawachi M, Morikawa M, Kazuta H, Shibata K, Ishida M, Kitagawa N, Matuso A, Kadota T (2004) Key parameters of sperm motion in relation to male fertility in rats given alphachlorohydrin or nitrobenzene. J Toxicol Sci 29:217-231.

Keeley T, McGreevy PD, O'Brien JK (2012) The effects of season and devil facial tumour disease on the reproductive physiology of the male Tasmanian devil (Sarcophilus harrisii). Reprod Fertil Dev 24:999-1007.

Kenagy GJ, Trombulak C (1986) Size and function of mammalian testes in relation to body size. J Mammal 67:1–22.

Kita S, Yoshioka M, Kashiwagi M, Ogawa S, Tobayama T (2001) Comparative external morphology of cetacean spermatozoa. Fisheries Sci 67:482-492.

Kraemer DC, Kuehl TJ (1980) Semen collection and evaluation of breeding soundness in nonhuman primates. Pp. 1134-1137 in D. A. Morrow, ed. Current therapy in theriogenology: diagnosis, treatment and prevention of reproductive diseases in animals. Saunders Co., Philadelphia, PA, USA.

LeMaître JF, Ramm SA, Barton RA, Stockley P (2009) Sperm competition and brain size evolution in mammals. J Evol Biol 22: 2215-2221.

Li HY, Zhang XF, Wang D, Chen DQ (2009) Ultrastructure of the spermatozoa of the Yangtze finless porpoise (Neophocaena phocaenoides asiaeorientalis). Anat Histol Embryol 38:300-304.

Lloyd S, Carrick F, Hall L (2002) Ultrastructure of the mature spermatozoon of the musky rat-kangaroo, Hypsiprymnodon moschatus (Potoroidae: Marsupialia). Acta Zool 83:167-174.

Lüke L, Vicens A, Serra F, Luque-Larena JJ, Dopazo H, Roldan ERS, Gomendio M (2011) Sexual Selection Halts the Relaxation of Protamine 2 among Rodents. PLoS ONE, 6:e29247

Lüke L, Vicens A, Tourmente M, Roldan ERS (2014) Evolution of protamine genes and changes in sperm head phenotype in rodents. Biol Reprod 90:67.

MacLeod CD (2010) The relationship between body mass and relative investment in testes mass in cetaceans: Implications for inferring interspecific variations in the extent of sperm competition. Mar Mammal Sci 26:370–380.

Marston, JH, Chang MC (1966) The morphology and timing of fertilization and early cleavage in the Mongolian gerbil and Deer mouse. J Embryol exp Morph 15:169-191.

Meisner AD, Klaus AV, O'Leary MA (2005) Sperm head morphology in 36 species of artiodactylans, perissodactylans, and cetaceans (Mammalia). J Morph, 263:179–202.

Miller DL, Styer EL, Decker SJ, Robeck TR (2002) Ultrastructure of the spermatozoa from three odontocetes: a killer whale (Orcinus orca), a Pacific white-sided dolphin (Lagenorhynchus obliquidens) and a beluga (Delphinapterus leucas). Anat Histol Embryol 31:158-168.

Neuenhagen C, Hartman MG, Greven H (2007) Histology and morphometrics of testes of the white-sided dolphin (Lagenorhynchus acutus) in bycatch samples from the northeastern Atlantic. Mammal Biol 72:283-298.

Paris DB, Taggart DA, Shaw G, Temple-Smith PD, Renfree MB (2005) Changes in semen quality and morphology of the reproductive tract of the male tammar wallaby parallel seasonal breeding activity in the female. Reproduction 130:367-378.

Plön S, Bernard RTF (2006) A review of spermatozoan morphology in Cetacea with new data for the genus Kogia. J Zool 269:466-473

Ramm SA, Oliver PL, Ponting CP, Stockley P, Emes RD (2008) Sexual selection and the adaptive evolution of mammalian ejaculate proteins. Mol Biol Evol 25: 207.

Ramm SA, Parker GA, Stockley P (2005) Sperm competition and the evolution of male reproductive anatomy in rodents. Proc R Soc B, 272:949–955.

Rose RW, Nevison CM, Dixson AF (1997) Testes weight, body weight and mating systems in marsupials and monotremes. J Zool 243:523–31.

Slott PA, Liu MH, Tavoloni N (1990) Origin, pattern, and mechanism of bile duct proliferation following biliary obstruction in the rat. Gastroenterology 99:466–477.

Taggart DA, Breed WG, Temple-Smith PD, Purvis A, Shimmin G (1998) Reproduction, mating strategies and sperm competition in marsupials and monotremes. In: Birkhead, T. R. and Møller, A. P. (eds), Sperm competition and sexual selection. Academic Press, pp. 623:666.

Taggart DA, Leigh CM, Breed WG (1995) Ultrastructure and motility of spermatozoa in the male reproductive tract of perameloid marsupials. Reprod Fertil Dev 7:1141-56.

Trainor BC, Martin LB 2nd, Greiwe KM, Kuhlman JR, Nelson RJ (2006) Social and photoperiod effects on reproduction in five species of Peromyscus. General and comparative endocrinology 148:252-259.

Westgate A, Read A (2007) Reproduction in short-beaked common dolphins (Delphinus delphis) from the western North Atlantic. Mar Biol 150:1011–1024.

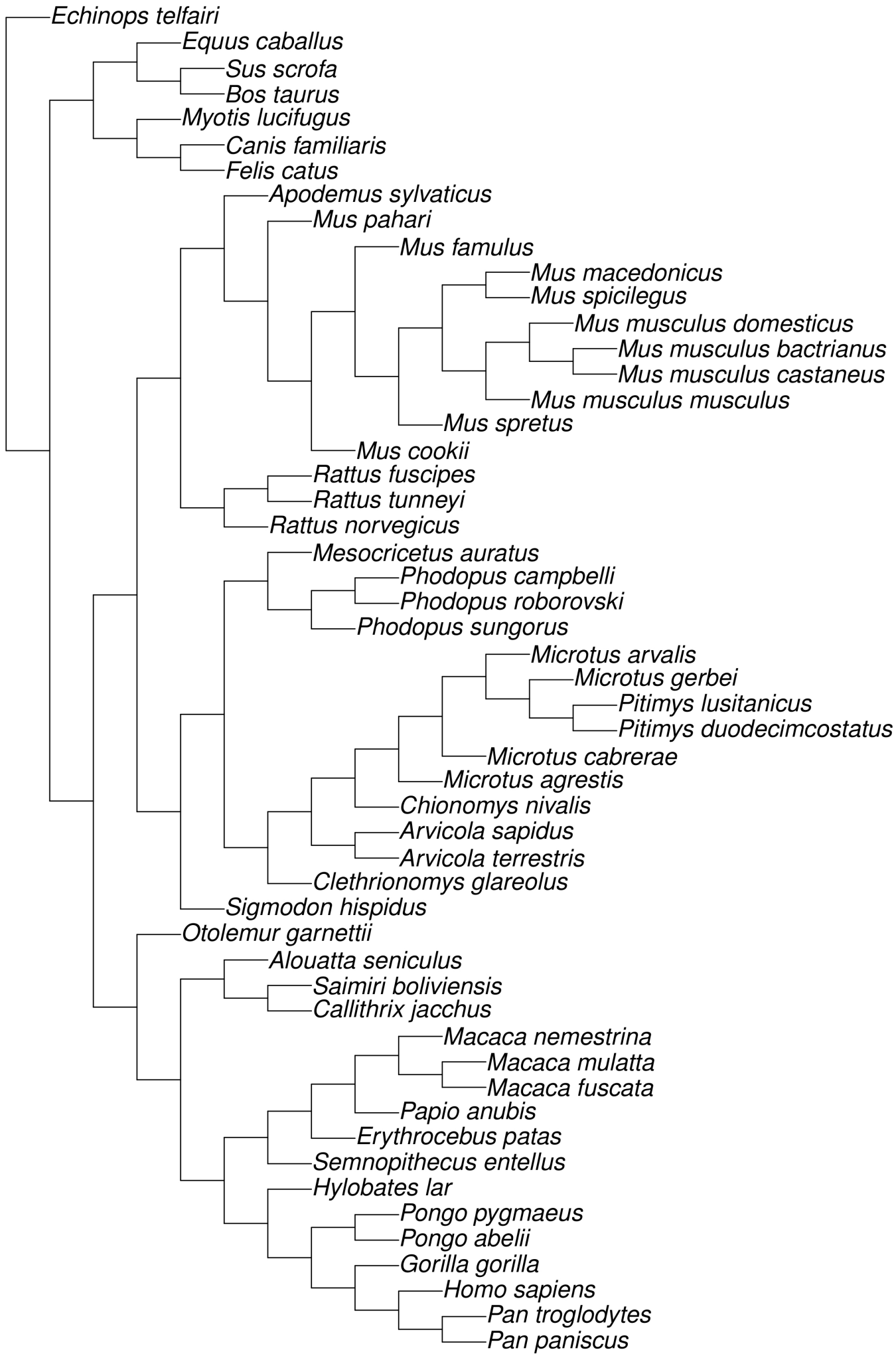
Wilkinson GS, McCracken GF (2003) Bats and balls: sexual selection and sperm competition in the Chiroptera. In: Kunz TH, Fenton BM (eds.) Bat ecology. University of Chicago Press (Chicago (IL)), pp. 128-151.

Wu PF, Chiang TA, Chen MT, Lee CP, Chen PH, Ko AM, Yang KJ, Chang PY, Ke DS, Ko YC (2010) A characterization of the antioxidant enzyme activity and reproductive toxicity in male rats following sub-chronic exposure to areca nut extracts. J Hazard Mater 178:541-546.

ANNEX III

CHAPTER 6: SELECTIVE CONSTRAINTS ON PROTAMINE 2 IN PRIMATES AND RODENTS

Figure S1. Phylogenetic tree constructed as consensus of phylogenetic data available in literature. References below.



References for suppl. Fig. S1

Adkins RM, Walton AH, Honeycutt RL (2003) Higher-level systematics of rodents and divergence time estimates based on two congruent nuclear genes. Mol Phylogenet Evol26:409–420.

Almeida FC, Bonvicino CR, Cordeiro-Estrela P (2007). Phylogeny and temporal diversification of Calomys (Rodentia, Sigmodontinae): Implications for the biogeographyof an endemic genus of the open/dry biomes of South America. Mol Phylogenet Evol42:449–466.

Álvarez A, Perez SI, Verzi DH (2011) Ecological and phylogenetic influence on mandible shape variation of South American caviomorph rodents (Rodentia: Hystricomorpha). Biol. J. Linn. Soc. 102:828-837.

Baena A, Mootnick AR, Falvo JV, Tsytskova AV, Ligeiro F, Diop OM, Brieva C, GagneuxP, O'Brien SJ, Ryder OA, Goldfeld AE (2005) Primate TNF promoters reveal markers ofphylogeny and evolution of innate immunity. PLoS One 2:e621.

Beck RM, Bininda-Emonds OR, Cardillo M, Liu FR, Purvis A (2006) A higher-level MRP supertree of placental mammals. BMC Evol Biol 6:93–107.

Bininda-Emonds ORP, Cardillo M, Jones KE, MacPhee RDE, Beck RMD, Grenyer R, Price SA, Vos RA, Gittleman JL, Purvis A (2007) The delayed rise of present-day mammals. Nature 446:507-512.

Böhm M, Mayhew PJ (2005) Historical biogeography and the evolution of the latitudinal gradient of species richness in the Papionini (Primata: Cercopithecidae). Biol J Linn Soc-Lond 85:235–246.

Borges BN, Paiva TS, Harada ML (2008) Evolution of the SEC1 gene in New World monkey lineages (Primates, Platyrrhini). Genet Mol Res 7:663–678.

Conroy CJ, Cook JA (2000) Molecular systematics of a holartic rodent (Microtus: Muridae). J Mammal 81:344–359.

Flynn JJ, Finarelli JA, Zehr S, Hsu J, Nedbal MA (2005) Molecular phylogeny of the Carnivora (Mammalia): assessing the impact of increased sampling on resolving enigmaticrelationships. Syst Biol 54:317–337.

Gomendio M, Tourmente M, Roldan ERS (2011) Why mammalian lineages respond differently to sexual selection: metabolic rate constrains the evolution of sperm size. Proc. R. Soc. Lond. B 278:3135-3141.

Gómez Montoto L, Magaña C, Tourmente M, Martín-Coello J, Crespo C, Luque-Larena JJ, Gomendio M, Roldan ERS (2011) Sperm competition, sperm numbers and sperm quality in muroid rodents. Plos One 6:e18173.

Johnson WE, Eizirik E, Pecon-Slattery J, Murphy WJ, Antunes A, Teeling E, O'Brien SJ(2006) The late Miocene radiation of modern Felidae: a genetic assessment. Science311:73–77.

Kjer KM, Honeycutt RL (2007) Site specific rates of mitochondrial genomes and the phylogeny of eutheria. BMC Evol Biol 7:8–17.

Lecompte E, Aplin K, Denys C, Catzeflis F, Chades M, Chevret P (2008) Phylogeny andbiogeography of African Murinae based on mitochondrial and nuclear gene sequences,with a new tribal classification of the subfamily. BMC Evol Biol 8:199–220.

Liu X, Wei F, Li M, Jiang X, Feng Z, Hu J (2004) Molecular phylogeny and taxonomy of wood mice (genus Apodemus Kaup, 1829) based on complete mtDNA cytochrome bsequences, with emphasis on Chinese species. Mol Phylogenet Evol 33:1–15.

Menzies JI (1996) A systematic revision of Melomys (Rodentia: Muridae) of New Guinea.Aust J Zool 44:367–426.

Michaux J, Chevret P, Renaud S (2007) Morphological diversity of Old World rats and mice(Rodentia, Muridae) mandible in relation with phylogeny and adaptation. J Zool Syst EvolRes 45:263–279.

Poux C, Douzery EJ (2004) Primate phylogeny, evolutionary rate variations, and divergence times: a contribution from the nuclear gene IRBP. Am J Phys Anthropol 124:1–16.

Prasad AB, Allard MW, Program NCS, Green ED (2008) Confirming the phylogeny of mammals by use of large comparative sequence data sets. Mol Biol Evol 25:1795-1808.

Prothero DR, Foss SE (2007) The Evolution of Artiodactyls (JHU Press, Baltimore, USA).

Sears KE, Finarelli JA, Flynn JJ, Wyss AR (2008) Estimating body mass in New World monkeys (Platyrrhini, Primates) with a consideration of the Miocene platyrrhine,Chilecebus carrascoensis. American Museum Novitates 3167:1–29.

Seddon JM, Baverstock PR (2000) Evolutionary Lineages of RT1.Ba in the Australian Rattus. Mol Biol Evol 17:768–772.

Springer MS, Murphy WJ (2007) Mammalian evolution and biomedicine: new views from phylogeny. Biol Rev 82:375–392.

Steiper ME, Ruvolo M (2003) New World monkey phylogeny based on X-linked G6PDDNA sequences. Mol Phylogenet Evol 27:121–130.

Steppan SJ, Adkins RM, Anderson J (2004) Phylogeny and divergence-date estimates of rapid radiations in Muroid rodents based on multiple nuclear genes. Syst Biol 53:533–553.

Steppan SJ, Adkins RM, Spinks PQ, Hale C (2005) Multigene phylogeny of the Old Worldmice, Murinae, reveals distinct geographic lineages and the declining utility ofmitochondrial genes compared to nuclear genes. Mol Phylogenet Evol 37:370–388.

Swann CA, Cooper SJ, Breed WG (2007) Molecular evolution of the carboxy terminal region of the zona pellucida 3 glycoprotein in murine rodents. Reproduction 133:697–708.

Valdespino C (2007) Physiological constraints and latitudinal breeding season in the Canidae. Physiol. Biochem. Zool. 80:580-591.

Xing J, Wang H, Han K, Ray DA, Huang CH, Chemnick LG, Stewart CB, Disotell TR,Ryder OA, Batzer MA (2005) A mobile element based phylogeny of Old World monkeys. Mol Phylogenet Evol 37:872–880.

Yoder AD, Yang Z (2004) Divergence dates for Malagasy lemurs estimated from multiplegene loci: geological and evolutionary context. Mol Ecol 13:757–773.

Table S1: Data included in study. HW= sperm head width, HL= sperm head length, TSL= total sperm length, BMASS= body mass, TMASS= testes mass

taxa	Order	Family	mature-Prm2 ω	cleaved-Prm2 ω	% arginine in mature-Prm2	HW/TSL	HL/TSL	HL/HW	BMASS (g, log)	TMASS (g, log)	Length cleaved-Prm2	Length mature-Prm2	Accession Prm2	References sperm dimensions	References body mass and testes mass
Alouatta_seniculus	Primates	Atelidae	1.38	0.26	50.00						48.00	52.00	X71335		
Apodemus_sylvaticus	Rodentia	Muridae	0.71	0.13	49.21	0.04	0.07	1.74	1.48	-0.02	44.00	63.00	FJ411393	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Arvicola_sapidus	Rodentia	Cricetidae	0.50	0.20	55.74	0.03	0.06	1.76	2.34	0.35	44.00	61.00	Lüke et al 2011	Lüke et al 2014	Gómez Montoto et al 2011
Arvicola_terrestris	Rodentia	Cricetidae	0.50	0.18	55.74				1.96	-0.39	44.00	61.00	Lüke et al 2011	Gómez Montoto et al 2011b	Lüke et al 2014
Bos_taurus	Artiodactyla	Bovidae	1.59	0.28	60.42	0.08	0.13	1.57	5.83	2.83	46.00	48.00	BK006493	Cummins & Woodall 1985; Gage 1998	Kenagy & Trombulak 1986
Callithrix_jacchus	Primates	Callithricidae	1.14	0.21	58.93		0.11		2.51	0.11	48.00	56.00	X85371	Cummins & Woodall 1985	Kenagy & Trombulak 1986
Canis_familiaris	Carnivora	Canidae	0.93	0.33	54.55	0.08	0.10	1.30	4.33	1.44	45.00	55.00	BK006497	Anderson et al 2005; Steklenev 1975	Woodall & Johnsten 1988
Chionomys_nivalis	Rodentia	Cricetidae	0.54	0.17	55.00	0.04	0.08	1.80	1.69	-0.01	44.00	60.00	Lüke et al 2011	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Clethrionomys_glareolus	Rodentia	Cricetidae	0.61	0.18	55.74	0.04	0.08	1.82	1.40	-0.37	44.00	61.00	Lüke et al 2011	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Echinops_telfairi	Afrosoricida	Tenrecidae									48.00	58.00	BK006491		
Equus_caballus	Perissodactyla	Equidae	1.30	0.34	61.40	0.05	0.12	2.12	5.67	2.62	44.00	57.00	BK006494	Cummins & Woodall 1985; Gage 1998	Kenagy & Trombulak 1986
Erythrocebus_patas	Primates	Cercopithecidae	1.16	0.26	54.55		0.08		4.11	0.86	48.00	55.00	AF195644	Anderson et al 2005	Dixson & Anderson 2004
Felis_catus	Carnivora	Felidae	1.27	0.45	53.19	0.04	0.09	2.05	3.49	0.37	45.00	47.00	BK006496	Terrell et al 2011	França & Godinho 2003
Gorilla_gorilla	Primates	Pongidae	1.76	0.31	48.15		0.08		5.13	1.37	48.00	54.00	X71336	Cummins & Woodall 1985	Kenagy & Trombulak 1986
Homo_sapiens	Primates	Hominidae	1.88	0.27	48.15		0.08		4.80	1.70	48.00	54.00	AF215713	Anderson et al 2005	Kenagy & Trombulak 1986
Hylobates_lar	Primates	Hylobatidae	1.61	0.29	50.00		0.11		3.74	0.74	48.00	54.00	X71339	Anderson et al 2005	Kenagy & Trombulak 1986
Macaca_fuscata	Primates	Cercopithecidae	1.32	0.27	58.18						48.00	55.00	AB101300		
Macaca_mulatta	Primates	Cercopithecidae	1.43	0.26	55.56	0.04	0.07	1.66	4.02	1.88	48.00	54.00	X71338	Anderson et al 2005; Cummins & Woodall 1985	Kenagy & Trombulak 1986
Macaca_nemestrina	Primates	Cercopithecidae	1.32	0.27	58.18		0.08		4.00	1.82	48.00	55.00	X71340	Anderson et al 2005	Kenagy & Trombulak 1986
Mesocricetus_auratus	Rodentia	Cricetidae	0.56	0.10	54.84	0.02	0.05	2.99	2.10	0.54	44.00	62.00	AF268204.1	Gage & Freckleton 2003	Kenagy & Trombulak 1986
Microtus_agrestis	Rodentia	Cricetidae	0.66	0.17	57.38		0.07		1.67	-0.10	44.00	61.00	Lüke et al 2011	Cummins & Woodall 1985	Kenagy & Trombulak 1986
Microtus_arvalis	Rodentia	Cricetidae	0.67	0.17	56.45	0.04	0.07	2.04	1.66	-0.32	42.00	62.00	Lüke et al 2011	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Microtus_cabrerae	Rodentia	Cricetidae	0.68	0.16	57.38	0.05	0.08	1.53	1.67	-0.81	42.00	61.00	Lüke et al 2011	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Microtus_gerbei	Rodentia	Cricetidae	0.65	0.17	58.06						44.00	62.00	Lüke et al 2011		
Mus_cookii	Rodentia	Muridae	0.68	0.13	50.79	0.03	0.07	2.00	1.37	-0.52	44.00	63.00	FJ411386	Lüke et al 2014	Gómez Montoto et al 2011
Mus_famulus	Rodentia	Muridae	0.74	0.12	52.38	0.03	0.07	1.97	1.44	-1.28	44.00	63.00	FJ411388	Lüke et al 2014	Gómez Montoto et al 2011
Mus_macedonicus	Rodentia	Muridae	0.74	0.12	50.79	0.03	0.07	2.06	1.30	-0.53	44.00	63.00	FJ411391	Lüke et al 2014	Gómez Montoto et al 2011
Mus_musculus_bactrianus	Rodentia	Muridae	0.76	0.12	50.79	0.03	0.06	2.22	1.26	-0.76	44.00	63.00	FJ411384	Lüke et al 2014	Gómez Montoto et al 2011
Mus_musculus_castaneus	Rodentia	Muridae	0.76	0.12	50.79	0.03	0.06	2.26	1.27	-1.12	44.00	63.00	FJ411385	Lüke et al 2014	Gómez Montoto et al 2011
Mus_musculus_domesticus	Rodentia	Muridae	0.74	0.12	50.79	0.03	0.06	2.03	1.34	-0.96	44.00	63.00	FJ411387	Lüke et al 2014	Gómez Montoto et al 2011
Mus_musculus_musculus	Rodentia	Muridae	0.76	0.12	50.79	0.03	0.08	2.52	1.34	-0.86	44.00	63.00	FJ411383	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Mus_pahari	Rodentia	Muridae	0.68	0.13	50.79	0.04	0.07	1.94	1.52	-0.89	44.00	63.00	FJ411389	Lüke et al 2014	Gómez Montoto et al 2011a
Mus_spicilegus	Rodentia	Muridae	0.71	0.13	52.38	0.03	0.08	2.47	1.26	-0.37	44.00	63.00	FJ411392	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Mus_spretus	Rodentia	Muridae	0.74	0.12	52.38	0.04	0.08	2.15	1.26	-0.52	44.00	63.00	FJ411390	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Myotis_lucifugus	Chiroptera	Vespertilionidae	0.81	0.28	62.26	0.04	0.08	2.39	0.83	-0.97	36.00	53.00	BK006495	Cummins & Woodall 2008	Hosken 1997
Otolemur_garnettii	Primates	Lemuridae	2.08	0.27	53.23						40.00	62.00	BK006492		
Pan_paniscus	Primates	Pongidae	1.75	0.30	48.15		0.07		4.59	2.13	48.00	54.00	X71334	Anderson et al 2005	Dixson & Anderson 2004
Pan_troglodytes	Primates	Pongidae	1.65	0.31	48.15		0.08		4.65	2.07	48.00	54.00	NM_001009084	Cummins & Woodall 1985	Kenagy & Trombulak 1986
Papio_anubis	Primates	Cercopithecidae	1.41	0.26	58.18		0.06		4.42	1.89	45.00	55.00	101009198.00	Anderson et al 2005	Dixson & Anderson 2004
Phodopus_campbelli	Rodentia	Cricetidae	0.64	0.14	50.00	0.03	0.06	2.46	1.69	0.29	40.00	62.00	Lüke et al 2011	Lüke et al 2014	
Phodopus_roborovski	Rodentia	Cricetidae	0.67	0.19	50.79	0.03	0.06	2.21	1.41	0.03	40.00	63.00	Lüke et al 2011	Lüke et al 2014	Ramm et al 2008
Phodopus_sungorus	Rodentia	Cricetidae	0.64	0.14	49.21	0.02	0.06	2.48	1.66	0.02	40.00	63.00	Lüke et al 2011	Gage & Freckleton 2003; Gage 1998	Hoffman 1979
Pitimys_duodecimcostatus	Rodentia	Cricetidae	0.65	0.17	58.06	0.05	0.08	1.59	1.44	-1.08	44.00	62.00	Lüke et al 2011	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Pitimys_lusitanicus	Rodentia	Cricetidae	0.65	0.16	58.06	0.04	0.07	1.84	1.27	-0.94	44.00	62.00	Lüke et al 2011	Gómez Montoto et al 2011b	Gómez Montoto et al 2011a
Pongo_abelii	Primates	Pongidae	1.66	0.28	44.44						48.00	54.00	100446402.00		
Pongo_pygmaeus	Primates	Pongidae	1.66	0.28	44.44		0.08		4.87	1.55	48.00	54.00	X71337	Cummins & Woodall 1985	Kenagy & Trombulak 1986
Rattus_fuscipes	Rodentia	Muridae	0.73	0.14	49.18		0.07		2.04	0.63	44.00	61.00	AF268201	Cummins & Woodall 1985	Breed & Taylor 2000
Rattus_norvegicus	Rodentia	Muridae	0.72	0.17	48.33		0.06		2.58	0.49	44.00	60.00	NM_012873.1	Cummins & Woodall 1985	Kenagy & Trombulak 1986; Wu et al 2010
Rattus_tunneyi	Rodentia	Muridae	0.72	0.17	50.82		0.11		2.39	0.69	44.00	61.00	AF268199	Olds et al	Breed 1997
Saimiri_boliviensis	Primates	Cebidae	0.96	0.24	60.71						48.00	56.00	101051182.00		
Semnopithecus_entellus	Primates	Cercopithecidae	0.98	0.25	56.36				4.27	1.05	48.00	55.00	AF195642		Harrison & Lewis 1986
Sigmodon_hispidus	Rodentia	Cricetidae	0.50	0.14	50.00	0.04	0.07	1.91	2.35	0.24	42.00	62.00	EU980396	Cummins & Woodall 1985	Kenagy & Trombulak 1986
Sus_scrofa	Artiodactyla	Suidae	1.55	0.24	60.42	0.09	0.16	1.70	4.60	2.11	44.00	48.00	NM_214252	Cummins & Woodall 1985; Gage 1998	Almeida et al 2006

References

Almeida FF, Leal MC, França LR (2006) Biol Reprod 75:792–799. **Cummins JM, Woodall PF** (1985) J Reprod Fertil 75:153–175. **Dixson AF, Anderson MJ** (2004) Physiol Behav 83:361–371. **Gage MJG** (1998) Proc Roy Soc Lond B Bio 265:97–103. **Gage MJG, Freckleton R** (2003) Proc Roy Soc Lond B 270:625–632. **L Gómez-Montoto, ERS. Roldan, M Gomendio**, unpublished data. **Gómez Montoto L, Magaña C, Tourmente M, Martín-Coello J, Crespo C et al.** (2011) PLoS ONE 6: e18173. **Gómez Montoto L, Varea Sánchez M, Tourmente M, Martín-Coello J, Luque-Larena JJ, Gomendio M, Roldan ERS** (2011b) Reproduction 142:819–830. **Harrison RM, Lewis RW** (1986) in Dukelow WR, Erwin J (eds): Comparative Primate Biology. New York, Liss, vol 3, pp 101–148. **Kenagy GJ, Trombulak** C (1986) J Mammal 67:1–22. **Lüke L, Vicens A, Serra F, Luque Larena JJ, Dopazo H, Roldan ERS, Gomendio M** (2011) PLoS ONE, 6:e29247. **Lüke L, Vicens A, Tourmente M, Roldan ERS** (2014) Biol Reprod 90:67. **Ramm SA, Oliver PL, Ponting CP, Stockley P, Emes RD** (2008) Mol Biol Evol 25: 207. **Ramm SA, Parker GA, Stockley** P (2005) Proc R Soc B, 272:949–955. **Slott PA, Liu MH, Tavoloni N** (1990) Gastroenterology 99:466–477. **Wu PF, Chiang TA, Chen MT, Lee CP, Chen PH, Ko AM, Yang KJ, Chang PY, Ke DS, Ko YC** (2010) J Hazard Mater 178:541-546.

Table S2. Phylogenetically controlled regression analyses (PGLS). The superscripts following the λ value indicate significance levels (n.s., p > 0.05; * p < 0.05) in likelihood ratio tests against models with λ = 0 (first superscript) and λ = 1 (second superscript). Only significant results and trends are shown in this table for non significant PGLS results see Table S4. Abbreviations: n = number of species in analysis. Significant regression results are shown in boldface.

Clade	dependent value	independent value	n	slope	t	R2	λ	p	
cleaved-Prm2									
Primates	cleaved-Prm2 ω	log body mass	12	0.02	0.23	0.14	1(ns,ns)	0.82	
		log testes mass		0.60	0.83			0.43	
Rodents	cleaved-Prm2 ω	log body mass	28	0.00	0.27	0.01	1(ns,ns)	0.79	
		log testes mass		0.00	-0.35			0.73	
Primates	relative head length	cleaved-Prm2 ω	11	0.02	0.07	0.00	1(ns,ns)	0.94	
Rodents	relative head length	cleaved-Prm2 ω	26	0.18	1.83	0.12	0.41(ns,ns)	0.08	
Rodents	**relative head width**	**cleaved-Prm2 ω**	**22**	**0.14**	**2.33**	**0.20**	**0.91(*,ns)**	**0.03**	*
Rodents	**sperm head elongation**	**cleaved-Prm2 ω**	**22**	**-8.07**	**-3.11**	**0.32**	**0.91(*,ns)**	**0.00**	**
mature-Prm2									
Primates	mature-Prm2 ω	log body mass	12	0.02	1.19	0.27	0.86(ns,ns)	0.26	
		log testes mass		0.00	0.36			0.73	
Rodents	mature-Prm2 ω	log body mass	28	-0.03	-1.80	0.17	1(*,ns)	0.09	
		log testes mass		-0.01	-0.30			0.77	
Primates	Arginine content (mature-Prm2)	log body mass	12	-1.32	-1.49	0.21	1(*,ns)	0.17	
		log testes mass		0.47	0.48			0.65	
Rodents	Arginine content (mature-Prm2)	log body mass	28	-0.02	-0.36	0.03	1(*,ns)	0.72	
		log testes mass		-0.02	-0.52			0.61	
Primates	relative head length	mature-Prm2 ω	10	-0.02	-0.82	0.07	1(*,ns)	0.43	
Rodents	relative head length	mature-Prm2 ω	26	0.03	1.15	0.05	0(ns,ns)	0.26	
Rodents	relative head width	mature-Prm2 ω	22	0.03	0.94	0.04	0.96(*,ns)	0.36	
Rodents	sperm head elongation	mature-Prm2 ω	22	-1.08	-0.83	0.03	1(*,ns)	0.41	

ANNEX IV

CHAPTER 7: SEXUAL SELECTION ON PROTAMINE AND TRANSITION NUCLEAR PROTEIN EXPRESSION IN MOUSE SPECIES

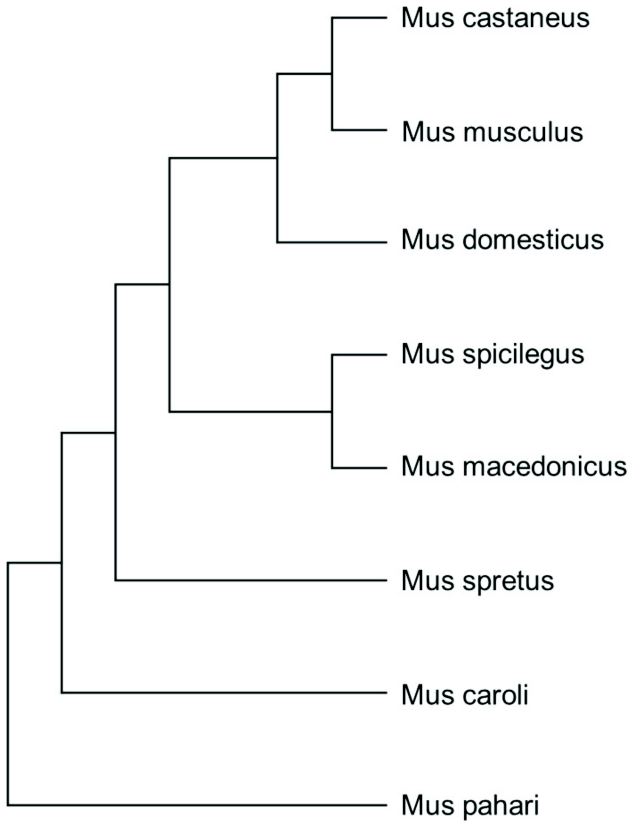


Figure S1. Tree of species included in this study.

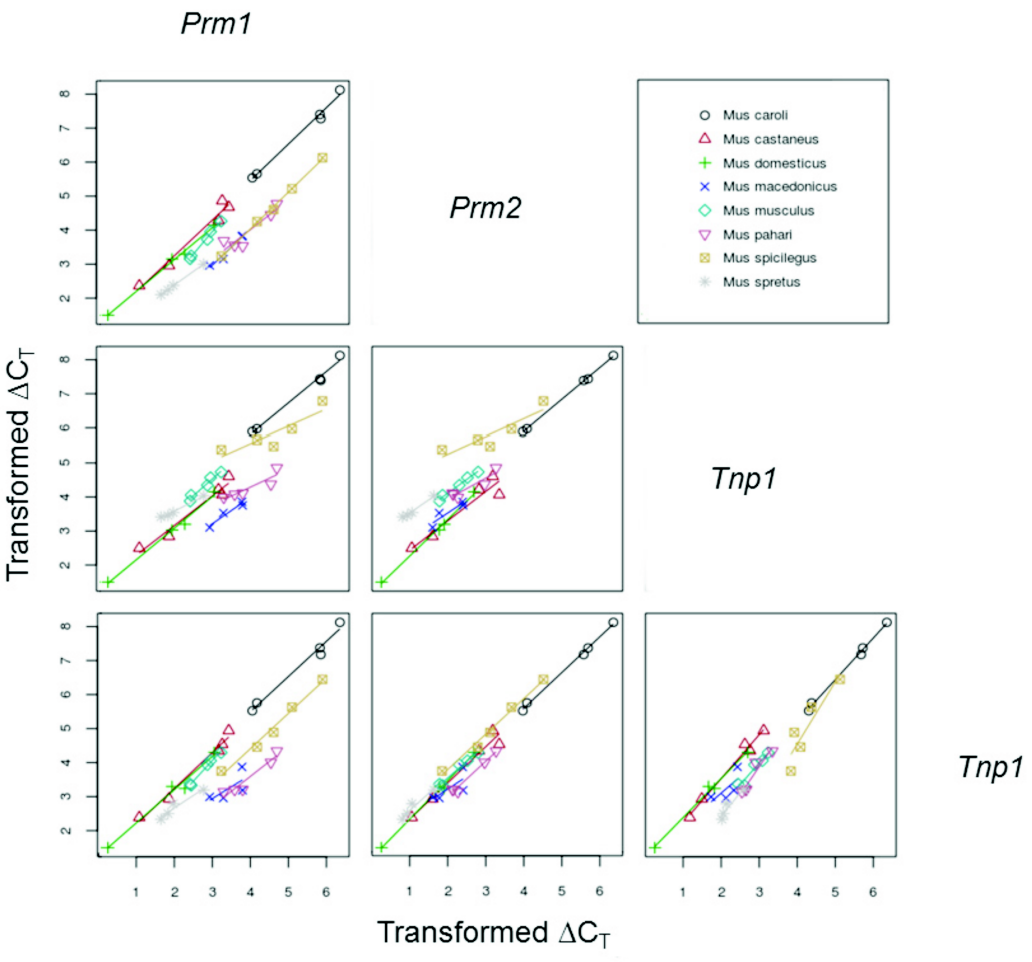


Figure S2. Gene expression correlation between transition proteins (*Tnp*) and protamines (*Prm*).

Table S1. Primer sequences used in quantitative PCR.

		Primer sequence	amplicon size (bases)
Prm1	fw	5'-AGGCGAAGATGTCGCAGACG-3'	87
	rv	3'-CCTTATGGTGTATGAGCGGCGG-5'	
Prm2	fw	5'-ACAAGAGGCGTCGGTCATGC-3'	125
	rv	3'-GTGCCTCCTACATTTCCTGCACC-5'	
Tnp1	fw	5'-TGAGGAGAGGCAAGAACCGAGC-3'	107
	rv	3'-TGCATCATCGCCCCGTTTCC-5'	
Tnp2	fw	5'-AGCTGCCCCAAGAACAGGAAGACC-3'	93
	rv	3'-ACGCCTCTTAGCTCTGTGAGTCCG-5'	
18srRNA	fw	5'-TGCAATCCCCGATCCCCATCAC-3'	174
	rv	3'-AGAGGGACAAGTGGCGTTCAGC-5'	

Table S2. Protamine and transition protein expression data. Results are normalized and transformed expression level ranges for each species (ΔC_T), individual variabilty for species (coefficient of variation, CV), species median and variabilty of expression for each gene (gene CV). Species are ordered by relative testes mass (ascending).

		expression (ΔC_T)		
gene	species	range	CV	median
Prm1	*Mus castaneus*	1.08 - 3.43	0.40	3.16
	Mus pahari	3.30 - 4.77	0.15	3.80
	Mus domesticus	0.25 - 3.05	0.63	2.11
	Mus musculus	2.44 - 3.23	0.13	2.87
	Mus caroli	4.05 - 6.35	0.20	5.83
	Mus spretus	1.65 - 2.77	0.24	1.90
	Mus macedonicus	2.93 - 3.80	0.12	3.45
	Mus spicilegus	3.24 - 5.90	0.22	4.61
		gene CV	0.37	
Prm2	*Mus castaneus*	2.37 - 4.68	0.29	4.29
	Mus pahari	3.53 - 4.77	0.14	3.69
	Mus domesticus	1.50 - 4.14	0.37	3.22
	Mus musculus	3.16 - 4.26	0.13	3.72
	Mus caroli	5.54 - 8.12	0.17	7.28
	Mus spretus	2.10 - 3.00	0.16	2.31
	Mus macedonicus	2.96 - 3.84	0.13	3.49
	Mus spicilegus	3.23 - 6.13	0.23	4.60
		gene CV	0.36	
Tnp1	*Mus castaneus*	1.19 - 3.75	0.42	2.96
	Mus pahari	2.87 - 3.85	0.12	3.01
	Mus domesticus	0.05 - 3.05	0.72	1.88
	Mus musculus	2.75 - 3.71	0.12	3.27
	Mus caroli	5.03 - 7.54	0.18	6.71
	Mus spretus	2.22 - 2.92	0.13	2.32
	Mus macedonicus	1.87 - 2.72	0.16	2.48
	Mus spicilegus	4.45 - 6.03	0.13	4.76
		gene CV	0.46	
Tnp2	*Mus castaneus*	1.45 - 3.97	0.38	3.38
	Mus pahari	2.19 - 3.37	0.21	2.26
	Mus domesticus	0.57 - 3.37	0.54	2.32
	Mus musculus	2.38 - 3.33	0.15	2.98
	Mus caroli	4.45 - 7.11	0.19	6.18
	Mus spretus	1.39 - 2.25	0.21	1.70
	Mus macedonicus	2.01 - 2.91	0.18	2.14
	Mus spicilegus	2.79 - 5.46	0.25	3.92
		gene CV	0.46	

Table S3. Pearson correlations between protamines and transition proteins, with species samples sizes (n) and p-values (p).

Mus spretus

Pearson r	*Tnp2*	*Tnp1*	*Prm2*	*Prm1*
Tnp2				
Tnp1	0.944			
Prm2	0.970	0.994		
Prm1	0.965	0.994	1.000	

n \ p	*Tnp2*	*Tnp1*	*Prm2*	*Prm1*
Tnp2		0.056	0.030	0.035
Tnp1	4		0.006	0.006
Prm2	4	4		0.000
Prm1	4	4	4	

Mus spicilegus

Pearson r	*Tnp2*	*Tnp1*	*Prm2*	*Prm1*
Tnp2				
Tnp1	0.913			
Prm2	0.994	0.901		
Prm1	0.992	0.881	0.999	

n \ p	*Tnp2*	*Tnp1*	*Prm2*	*Prm1*
Tnp2		0.030	0.001	0.001
Tnp1	5		0.037	0.049
Prm2	5	5		0.000
Prm1	5	5	5	

Mus pahari

Pearson r	*Tnp2*	*Tnp1*	*Prm2*	*Prm1*
Tnp2				
Tnp1	0.953			
Prm2	0.988	0.926		
Prm1	0.962	0.906	0.918	

n \ p	*Tnp2*	*Tnp1*	*Prm2*	*Prm1*
Tnp2		0.012	0.002	0.009
Tnp1	5		0.024	0.034
Prm2	5	5		0.028
Prm1	5	5	5	

Mus musculus

Pearson r	*Tnp2*	*Tnp1*	*Prm2*	*Prm1*
Tnp2				
Tnp1	0.969			
Prm2	0.988	0.992		
Prm1	0.994	0.978	0.995	

n \ p	*Tnp2*	*Tnp1*	*Prm2*	*Prm1*
Tnp2		0.006	0.002	0.001
Tnp1	5		0.001	0.004
Prm2	5	5		0.000
Prm1	5	5	5	

Mus domesticus

Pearson r	*Tnp2*	*Tnp1*	*Prm2*	*Prm1*
Tnp2				
Tnp1	0.996			
Prm2	0.997	0.998		
Prm1	0.990	0.996	0.998	

n \ p	*Tnp2*	*Tnp1*	*Prm2*	*Prm1*
Tnp2		0.004	0.003	0.010
Tnp1	4		0.002	0.004
Prm2	4	4		0.002
Prm1	4	4	4	

Mus castaneus

Pearson r	*Tnp2*	*Tnp1*	*Prm2*	*Prm1*
Tnp2				
Tnp1	0.992			
Prm2	0.981	0.955		
Prm1	0.991	0.980	0.984	

n \ p	*Tnp2*	*Tnp1*	*Prm2*	*Prm1*
Tnp2		0.001	0.003	0.001
Tnp1	5		0.011	0.003
Prm2	5	5		0.002
Prm1	5	5	5	

Mus macedonicus

Pearson r	*Tnp2*	*Tnp1*	*Prm2*	*Prm1*
Tnp2				
Tnp1	0.703			
Prm2	0.726	0.919		
Prm1	0.683	0.971	0.983	

n \ p	*Tnp2*	*Tnp1*	*Prm2*	*Prm1*
Tnp2		0.297	0.274	0.317
Tnp1	4		0.081	0.029
Prm2	4	4		0.017
Prm1	4	4	4	

Mus caroli

Pearson r	*Tnp2*	*Tnp1*	*Prm2*	*Prm1*
Tnp2				
Tnp1	0.998			
Prm2	0.998	1.000		
Prm1	0.990	0.995	0.995	

n \ p	*Tnp2*	*Tnp1*	*Prm2*	*Prm1*
Tnp2		0.000	0.000	0.001
Tnp1	5		0.000	0.000
Prm2	5	5		0.000
Prm1	5	5	5	

Table S4. Protamine and transition protein relative expression data. Results are presented as ratios or proportions of expression levels of one gene in relation to others after expression was normalized and transformed for each species (ΔC_T). Individual variabilty for each species (coefficient of variation, CV), species median and variablity medians of expression for ratios and percentages (ratio CV or Proportion CV) are given. Species are ordered by relative testes mass (ascending).

		relative expression		
ratio or proportion	Species	range	CV	median
Tnp1/Tnp2	*Mus castaneus*	0.79 - 0.93	0.07	0.83
	Mus pahari	1.09 - 1.33	0.09	1.13
	Mus domesticus	0.76 - 0.92	0.09	0.81
	Mus musculus	1.10 - 1.24	0.05	1.14
	Mus caroli	1.06 - 1.11	0.02	1.07
	Mus spretus	1.28 - 1.59	0.10	1.38
	Mus macedonicus	0.92 - 1.17	0.13	1.05
	Mus spicilegus	1.10 - 1.59	0.17	1.16
		ratio CV	0.18	
Prm1/Prm2	*Mus castaneus*	0.46 - 0.74	0.18	0.67
	Mus pahari	0.90 - 1.08	0.07	1.01
	Mus domesticus	0.61 - 0.74	0.09	0.65
	Mus musculus	0.75 - 0.77	0.01	0.76
	Mus caroli	0.73 - 0.80	0.04	0.78
	Mus spretus	0.78 - 0.92	0.07	0.82
	Mus macedonicus	0.99 - 1.04	0.03	0.99
	Mus spicilegus	0.96 - 1.00	0.02	0.98
		ratio CV	0.17	
Prm/Tnp	*Mus castaneus*	1.08 - 1.35	0.09	1.24
	Mus pahari	1.31 - 1.42	0.03	1.38
	Mus domesticus	1.13 - 1.31	0.07	1.27
	Mus musculus	1.04 - 1.08	0.01	1.06
	Mus caroli	0.99 - 1.02	0.01	1.00
	Mus spretus	1.03 - 1.12	0.04	1.05
	Mus macedonicus	1.53 - 1.58	0.06	1.49
	Mus spicilegus	0.89 - 1.09	0.07	1.05
		ratio CV	0.15	
Prm2/Tnp	*Mus castaneus*	0.62 - 0.90	0.16	0.75
	Mus pahari	0.66 - 0.73	0.04	0.68
	Mus domesticus	0.65 - 0.77	0.09	0.77
	Mus musculus	0.60 - 0.61	0.01	0.61
	Mus caroli	0.55 - 0.58	0.02	0.56
	Mus spretus	0.56 - 0.59	0.02	0.58
	Mus macedonicus	0.68 - 0.79	0.07	0.74
	Mus spicilegus	0.45 - 0.54	0.08	0.53
		ratio CV	0.14	
Prm2/Prm	*Mus castaneus*	0.58 - 0.69	0.07	0.60
	Mus pahari	0.48 - 0.53	0.03	0.50
	Mus domesticus	0.58 - 0.62	0.04	0.61
	Mus musculus	0.56 - 0.57	0.01	0.57
	Mus caroli	0.55 - 0.58	0.02	0.56
	Mus spretus	0.52 - 0.56	0.03	0.55
	Mus macedonicus	0.49 - 0.50	0.01	0.50
	Mus spicilegus	0.50 - 0.51	0.01	0.50
		Proportion CV	0.08	
Prm2/(Prm+Tnp)	*Mus castaneus*	0.30 - 0.39	0.11	0.33
	Mus pahari	0.28 - 0.31	0.03	0.29
	Mus domesticus	0.31 - 0.34	0.06	0.34
	Mus musculus	0.29 - 0.30	0.01	0.29
	Mus caroli	0.28 - 0.29	0.02	0.28
	Mus spretus	0.27 - 0.29	0.02	0.28
	Mus macedonicus	0.29 - 0.31	0.03	0.30
	Mus spicilegus	0.24 - 0.26	0.04	0.26
		Proportion CV	0.09	

ANNEX V

CHAPTER 8: PROTAMINES AND SPERM HEAD PHENOTYPE: A COMPARATIVE ANALYSIS

Figure S1. Phylogenetic tree used in PGLS analyses.

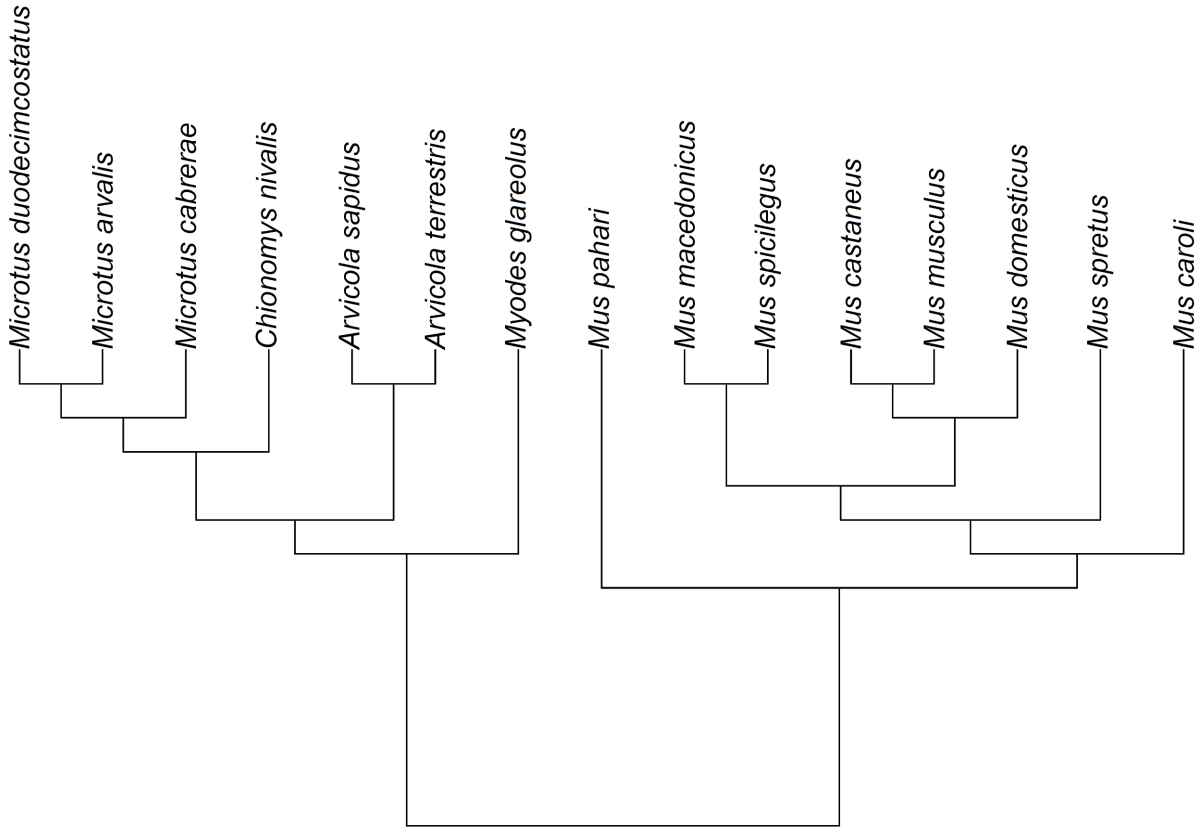


Table S1. Primers used in qPCR. Abbreviations: fw = forward primer, rv = reverse primer.

Primer name	Sequence	spp
Protamine 1 fw	CCAGATACCGCTRCCGCAGC	All voles
Protamine 1 rv1	CTCATGGTGTATGTGCGGCGG	*A sapidus, A terrestris, M cabrerae*
Protamine 1 rv2	AGCACCTTCGCCTCCTCCTCC	*M glareolus, C nivalis, M duodecimcostatus, M arvalis*
Protamine 2 fw	CATCAYAGACGGCGCTGCTCGC	All voles
Protamine 2 rv	CTACGATGCCGCCTCCTGTGG	All voles

Table S2. Phylogenetically controlled regression analyses (PGLS). The superscripts following the λ value indicate significance levels (n.s., p > 0.05; * p < 0.05) in likelihood ratio tests against models with λ = 0 (first superscript) and λ = 1 (second superscript). Abbreviations: n = number of species in analysis, HL = sperm head length, HW = sperm head width, HA = sperm head area.

		PGLS analysis in voles							PGLS analysis in mice					
dependent variable	independent variable	n	slope	F	p		R2	λ	n	slope	F	p		R2	λ
Protamine1 promoter	body mass (log)		0.11	5.17	0.003					−0.025	−1.59	0.18			
	testes mass (log)	7	−0.067	−3.75	0.013	*	0.84	0(ns,*)	7	−0.008	−0.99	0.38		0.39	0(ns,*)
Protamine2 promoter	body mass (log)		0.012	0.37	0.73					0.11	4.18	0.01			
	testes mass (log)	7	−0.01	−0.57	0.6		0.08	1(ns,ns)	7	0.02	1.33	0.255		0.87	0(ns,*)
Protamine1 expression	body mass (log)		−5.49	−5.46	0.005					−2.44	−0.41	0.7			
	testes mass (log)	7	2.82	3.31	0.029	*	0.88	0(ns,ns)	8	0.26	0.11	0.92		0.04	1(ns,ns)
Protamine2 expression	body mass (log)		−9.32	−5.34	0.0059					−7.34	−11.1	0.322			
	testes mass (log)	7	5.14	3.47	0.25	*	0.87	0(ns,ns)	8	−1.8	−0.66	0.54		0.21	1(ns,ns)
Protamine expression ratio	body mass (log)		0.24	1.17	0.16					0.261	7.44	0			
	testes mass (log)	7	−0.22	−2.69	0.055	.	0.64	0.99(fixed)	8	0.124	6.02	0.002	**	0.93	0(ns,*)
Relative HL	body mass (log)		−0.03	−4.2	0.013					0.016	1.82	0.12			
	testes mass (log)	7	0.01	0.9	0.42		0.85	0(ns,ns)	8	0.025	4.81	0.004	**	0.82	0(ns,ns)
Relative HW	body mass (log)		−0.012	−0.86	0.44					0.017	1.78	0.13			
	testes mass (log)	7	−0.01	−0.13	0.9		0.27	0.78(fixed)	8	0.012	2.69	0.043	*	0.62	0.78(fixed)
Relative HA	body mass (log)		−0.15	−3.01	0.04					0.258	3.84	0.012			
	testes mass (log)	7	0.07	1.75	0.16		0.69	0(ns,ns)	8	0.07	2.49	0.05	.	0.77	0.81(ns,ns)
Protamine1 expression	Protamine 1 promoter	7	−43.45	−5.59	0.002	**	0.86	0(ns,ns)	7	−191.94	−3.28	0.022	*	0.68	0(ns,*)
Protamine2 expression	Protamine 1 promoter	7	−64.71	−3.11	0.026	*	0.66	0(ns,*)	7	−104.73	−1.88	0.11		0.42	0(ns,ns)
Protamine expression ratio	Protamine 1 promoter	7	1.05	0.71	0.51		0.09	0.99(fixed)	7	−2.97	−1.8	0.13		0.39	0.99(fixed)
Protamine1 expression	Protamine 2 promoter	7	−47.4	−1.8	0.13		0.39	1(ns,ns)	7	61.53	3.04	0.028	*	0.65	0(ns,ns)
Protamine2 expression	Protamine 2 promoter	7	−61.19	−0.98	0.37		0.16	0.83(ns,ns)	7	33.2	1.78	0.13		0.39	0(ns,ns)
Protamine expression ratio	Protamine 2 promoter	7	0.74	0.41	0.7		0.03	0(ns,ns)	7	1.42	1.54	0.18		0.32	0.89(ns,ns)
Relative HL	Protamine1 expression	7	0.0053	3	0.03	*	0.64	1(*,ns)	8	−0.001	−0.08	0.94		0.001	0(ns,ns)
Relative HW	Protamine1 expression	7	0.003	1.63	0.16		0.34	0.44(ns,ns)	8	−0.001	−0.1	0.92		0.001	0.85(ns,ns)
Relative HA	Protamine1 expression	7	0.032	3.34	0.021	*	0.69	1(ns,ns)	8	0.001	0.07	0.94		0.01	0.89(ns,ns)
Relative HL	Protamine2 expression	7	0.0029	3.47	0.018	*	0.707	0(ns,ns)	8	−0.001	0.84	0.43		0.1	0(ns,*)
Relative HW	Protamine2 expression	7	0.002	1.87	0.12		0.41	0(ns,ns)	8	−0.001	0.85	0.42		0.1	0.89(ns,ns)
Relative HA	Protamine2 expression	7	0.015	3.94	0.011	*	0.756	0(ns,ns)	8	−0.01	−0.73	0.49		0.08	0.89(*,ns)
Relative HL	Protamine expression ratio	7	−0.078	−2.6	0.048	*	0.57	0(ns,ns)	8	0.12	2.37	0.056	.	0.48	0.38(ns,ns)
Relative HW	Protamine expression ratio	7	−0.05	−1.82	0.4		0.13	0(ns,*)	8	0.08	2.6	0.041	*	0.52	0.92(ns,ns)
Relative HA	Protamine expression ratio	7	−0.414	−2.94	0.032	*	0.63	0(ns,ns)	8	0.7	2.83	0.03	*	0.57	0.86(ns,ns)

ANNEX VI

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1. List of published articles

Lüke L, Vicens A, Tourmente M, Roldan ERS (2014a) Evolution of protamine genes and changes in sperm head phenotype in rodents. Biol Reprod 90:67.

Lüke L, Campbell P, Sánchez MV, Nachman MW, Roldan ERS (2014b) Sexual selection on protamine and transition nuclear protein expression in mouse species. Proc R Soc B 281:2013-3359

3. List of articles submitted for publication

Lüke L, Tourmente M, Roldan ERS (0000a) Sexual selection of protamine 1 in mammals. *submitted*.

Lüke L, Tourmente M, Dopazo H, Serra F, Roldan ERS (0000b) Selective constraints on protamine 2 in primates and rodents. s*ubmitted*.

4. List of articles in preparation

Lüke L, Varea Sanchez M, Tourmente M, Ortiz D, Roldan ERS (0000c) Protamines and sperm head phenotype: A comparative analysis. *in preparation.*

ANNEX VII

Published papers:

BIOLOGY OF REPRODUCTION (2014) **90**(3):67, 1–8
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Evolution of Protamine Genes and Changes in Sperm Head Phenotype in Rodents[1]

Lena Lüke, Alberto Vicens, Maximiliano Tourmente, and Eduardo R.S. Roldan[2]

Reproductive Ecology and Biology Group, Museo Nacional de Ciencias Naturales (CSIC), Madrid, Spain

ABSTRACT

Little is known about the genetic basis of evolutionary changes in sperm phenotype. Postcopulatory sexual selection is associated with differences in protamine gene sequences and promoters and is a powerful force acting on sperm form and function, although links between protamine evolution and sperm phenotype are scarce. Protamines are involved in sperm chromatin condensation, and protamine deficiency negatively affects sperm morphology and male fertility, thus suggesting that they are important for sperm design and function. We examined changes in protamine genes and sperm phenotype in rodents to understand the role of sexual selection on protamine evolution and sperm design. We performed a genotype-phenotype association study using root-to-tip dN/dS (nonsynonymous/synonymous substitutions rate ratio) to account for evolutionary rates and phylogenetic generalized least squares analyses to compare genetic and morphometric data. Evolutionary rates of protamine 1 and the protamine 2 domain cleaved off during chromatin condensation correlated with head size and elongation. Protamine 1 exhibited restricted positive selection on some functional sites, which seemed sufficient to preserve its role in head design. The cleaved-protamine 2, whose relaxation is halted by sexual selection, seems to ensure small, elongated heads that would make sperm more competitive. No association existed between mature-protamine 2 and head phenotype, suggesting little involvement during chromatin condensation and a likely role maintaining the condensed state. Our results suggest that evolutionary changes in protamines could be related to complex developmental modifications in the sperm head. This represents an important step toward understanding the role of changes in gene coding sequences in the divergence of germ cell phenotype.

evolutionary rates, positive selection, protamines, sperm head, sperm phenotype

INTRODUCTION

Postcopulatory sexual selection, in the form of sperm competition, is an evolutionary force known to drive rapid adaptation of reproductive traits [1–4] and that has also been linked to rapid diversification of coding sequences of the so-called reproductive proteins [5, 6]. Reproductive proteins include those that act following copulation and that mediate gamete usage, storage, signal transduction, and fertilization [5]. Sperm competition occurs when females mate with more than one male during their receptive period, resulting in rival ejaculates competing for fertilization of the ova [1]. The diversity of male reproductive traits and the adaptive significance of differences in sperm form and function have been analyzed using comparative methods and, more recently, experimental evolution studies [4]. However, the genetic basis of evolutionary changes in sperm phenotype has, so far, received very limited attention in mammals.

The most widely recognized phenotypic response to an increased level of sperm competition is an increase in sperm numbers [2, 4], which enhances a male's chance of fertilization [2, 7, 8]. High sperm numbers may be achieved by an increase in the size of the testes relative to body mass (relative testes mass). Such an increase in relative testes mass is associated with sperm competition in a variety of taxa [2, 4, 9], including mammals [10], and is widely used as a proxy for sperm competition. Another important sperm trait found to be driven by sperm competition in mammals is sperm design (i.e., sperm dimensions and head shape) [11–14]. Sperm design is known to influence sperm function by affecting sperm swimming velocity in many taxa (reviewed in [15]). In mammals, sperm head shape and size may infuence hydrodynamic efficiency of the sperm cell and thereby affect swimming velocity [13, 16]. Sperm that have more elongated heads and heads that are smaller in relation to the length of the flagellum exhibit higher swimming velocity. A smaller and more elongated head is believed to produce less drag during swimming and is therefore thought to positively influence sperm velocity [17]. In rodents, spermatoza from many species display a hook in their rostral region; hook shape and size may also have an impact on sperm swimming velocity [18]. Altogether, the evidence available indicates that sperm head shape and size are important factors in sperm competition because they strongly influence how fast sperm may swim toward the ova.

Proteins of the reproductive system that affect crucial phenotypic traits are thought to experience rapid divergence in their gene sequences [5, 6]. Evidence for a link between elevated evolutionary rate and sexual selection is, however, limited. Studies that examined this relationship tested for correlations between evolutionary rate of the gene sequence and a proxy for sperm competition, that is, relative testes mass, number of mating partners, and level of sexual dimorphism (in primates) in different groups of species. Only four studies have found positive relationships between gene sequence divergence (evolutionary rate) and levels of sperm competition: SEMG2 [19], SVS2 [20], ADAM2 and ADAM18 [21], and zonadhesin [22]. Other studies suggest that in some genes positive selection may not be responsible for fast evolutionary rates but, instead, that such fast evolutionary rate may be due to a relaxation of functional constraints acting on gene sequences.

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[2]Correspondence: Eduardo R.S. Roldan, Reproductive Ecology and Biology Group, Museo Nacional de Ciencias Naturales (CSIC), c/José Gutierrez Abascal 2, 28006-Madrid (Spain).
E-mail: roldane@mncn.csic.es

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These studies identified negative relationships between gene sequence divergence and sperm competition [23, 24].

Protamines are nuclear proteins involved in sperm chromatin condensation. They replace histones and transition proteins during the process of sperm chromatin condensation that takes place in spermiogenesis [25]. In some eutherian mammals, two types of protamines are found: protamine 1 (PRM1) and protamine 2 (PRM2). While PRM1 is present in all mammals, PRM2 is only detected in sperm of primates, most rodents, and a subset of other placental mammals [26, 27]. In contrast to the *Prm1* gene, the *Prm2* gene codes for a precursor (hereafter, PRM2 precursor), which is processed in elongated spermatids by successive proteolytic cleavages. This results in the removal of about 40% of the protein N-terminal region (the removed domain is hereafter referred to as cleaved-PRM2) [25, 26]. The PRM2 form resulting after full cleavage (hereafter, mature-PRM2) consists of 63 amino acids in the mouse. The mature-PRM2 shows very similar structural and functional properties to PRM1 and is proposed to be the result of gene duplication, while cleaved-PRM2 shows no resemblance and might be of retroviral origin [24, 28].

It has been claimed that protamines are the fastest evolving reproductive proteins, with sexual selection being the underlying selective force [29]. Evidence of positive selection in PRM1 has been detected in primates [29, 30], but recent studies have revealed some contrasting results in other mammalian species. In closely related mouse species (genus *Mus*), no evidence of positive selection was found for *Prm1* and only weak positive selection for *Prm2* [31]. In a group of more diverse rodents (voles and hamsters), *Prm1* is conserved with signs of localized positive selection but no evidence of being sexually selected [24]. On the other hand, the *Prm2* gene sequence of voles and hamsters was found to be under relaxation, leading to degradation of the gene. This process is halted by sexual selection removing deleterious mutations in species with higher levels of sperm competition [24]. In closely related species, among which conservation of coding regions may exist, the evolution of gene regulatory regions may be an early stage in speciation. Among protamines, an association was found between the divergence of *Prm2* promoters and levels of sperm competition of mouse species [31]. Divergence in the *Prm2* promoter was also associated with sperm swimming velocity [31], which suggests that changes in regulatory regions could increase the efficiency of DNA condensation in the sperm head, thus, affecting head shape and size and, in turn, sperm performance.

The importance of protamines in sperm chromatin condensation and the influence of protein expression on sperm function have been shown in mouse and human models [26, 32–34]. Alterations in sperm protamine content can have major negative effects on sperm concentration, motility and sperm head morphology in men [33]. Haploinsufficiency of protamines in mice results in sperm morphological abnormalities, DNA damage, and decreases in sperm motility [35]. In particular, PRM2 deficiency has a negative impact on chromatin packaging and sperm head morphology [32]. Incorrect condensation of sperm chromatin results in larger heads as well as head abnormalities [36]. This further supports the idea that protamines are important in sperm head formation and function.

To understand the effect of sperm competition on reproductive trait evolution, both molecular changes and the phenotypic response should ideally be analyzed together. Here we analyze the effect of protamines on sperm head size by examining a possible association between divergence in gene sequences and various sperm head dimensions. In order to compare genetic and morphometric data, we employed methods used in previous studies to analyze genotype-phenotype correlations, that is, the phylogenetic generalized least squares (PGLS) approach (to account for phylogenetic bias in the correlation) using phylogenetic data and root-to-tip dN/dS (nonsynonymous/synonymous substitutions rate ratio, a measure of the evolutionary rate of gene sequences for each species) [37, 38]. Using these approaches, genetic-morphometric associations have been uncovered in studies of microcephaly genes and brain size [37], evolution of RUNX2 and face length [37], and the evolution of ASPM in both an increase and decrease of brain size [39].

We studied a group of rodent species belonging to the family Cricetidae (subfamilies Arvicolinae and Cricetinae) [24]. These species have a wide range of relative testes mass and, therefore, inferred levels of sperm competition and, unlike murids, they show a diverse range of sperm head sizes and a higher level of divergence in the protamine coding sequences. To incorporate the structural and functional differences of the two main PRM2 domains (cleaved-PRM2 and mature-PRM2) we analyzed their respective coding sequences separately to take into account possible differences in how they may affect sperm head size. The *Prm2* gene sequence in this group of species was shown to be under relaxation, which is halted in species with higher levels of sperm competition [24]. Species with high levels of sperm competition therefore show a lower evolutionary rate. Consequently, we expected a negative association between the evolutionary rate of *Prm2* and sperm head size. In addition, although PRM1 was not shown to be under postcopulatory sexual selection in these cricetid rodents, it was still expected to influence sperm head size based on its functional similarity with PRM2 and their joint action in sperm nucleus condensation; therefore, this gene was also included in the study.

MATERIALS AND METHODS

Ethics

All the procedures were carried out following Spanish Animal Protection Regulation RD1201/2005, which conforms to European Union Regulation 2003/65. The research protocol was approved by the Ethics Committee of the Spanish Research Council (CSIC). Wild specimens were captured with permits from the Comunidad de Madrid and the Junta de Castilla-León, Spain.

Animals

The study included 12 species of the family Cricetidae, eight of which belong to the subfamily Arvicolinae (*Arvicola sapidus, Arvicola terrestris, Clethrionomys glareolus, Chinomys nivalis, Microtus arvalis, Microtus cabrerae, Pitymys duodecimostatus, Pitymys lusitanicus*) and four to the subfamily Cricetinae (*Mesocricetus auratus, Phodopus sungorus*, *Phodopus campbelli, Phodopus roborovskii*) [40]. This group of species has experienced rapid evolutionary radiation and diversification [40], and shows different levels of sperm competition, as suggested by their differences in relative testes mass [24]. Individuals belonging to Arvicolinae were trapped in the field during the breeding season at different locations in Spain [41]. Individuals belonging to Cricetinae stem from laboratory strains purchased from commercial suppliers and were unrelated [24]. We obtained the gene sequences of at least four individuals per species to generate a consensus sequence. For assessments of sperm traits, sample size varied between three and eight individuals for each species. For all the species, size variance (coefficient of variation, CV) was much lower within species than between species (e.g., *Pitymys lusitanicus* ($n = 3$): CV head length = 0.04, CV sperm length = 0.04; all species: CV head length = 0.15, CV sperm length = 0.3). Males were kept in our animal facilities in individual cages under standard laboratory conditions in environmentally controlled rooms (20°C–24°C) on a 14L:10D photoperiod and provided with food and water ad libitum.

Protamine Sequences

Prm1 sequence for *P. sungorus* and *P. roborovskii* and *Prm1* and *Prm2* sequences for *M. auratus* were obtained from the literature [20, 42]. All the other nucleotide sequences were obtained through PCR amplification and sequencing.

DNA Isolation and Gene Amplification

Genomic DNA was extracted from frozen tissues using the E.Z.N.A Tissue DNA kit (Omega, Madrid, Spain) following the manufacturer's recommendations. Protamine sequences were amplified by polymerase chain reaction (PCR). PCR mixtures were prepared in a 50 μl volume containing PCR Gold buffer 1× (Roche, Barcelona, Spain), 2.5 mM $MgCl_2$ (Roche), 0.8 mM dNTPs mix supplying 0.2 mM of each deoxynucleotide triphosphate (Applied Biosystems, Barcelona, Spain), 0.3 mM of forward and reverse primers (Applied Biosystems), 2 units of Taq Gold DNA polymerase (Roche), and 20–100 ng/μl of genomic DNA template. All the PCRs were performed in a Veriti thermocycler (Applied Biosystems). The conditions of the thermocycler program consisted of 35–45 cycles with an initial denaturation of 95°C for 30–40 sec, an annealing stage at 52°C–62°C (depending on template and primers) for 40 sec, and an elongation stage at 72°C for 30–50 sec (depending on gene length). PCR primers were designed on the basis of protamine genomic sequences of other closely related rodent species accessible in the literature or in National Center for Biotechnology Information GeneBank. All the alignments were performed in BioEdit [43], and the most conserved segments within untranslated regions were chosen. When protamines of one or more individuals of each closely related group were sequenced, new specific primers on the basis of these sequences were designed to ensure efficient PCR performance. The primer sequences can be found in Supplemental Table S1 (Supplemental Data are available online at www.biolreprod.org). PCR products were purified by using the E.Z.N.A. Cycle Pure kit (Omega). In cases in which additional nonspecific bands were obtained after separation in a 1.5% agarose gel, bands of about 600 bp size for *Prm2* and about 300 bp size for *Prm1* were extracted with E.Z.N.A. Gel Extraction Kit (Omega). The purified products were sequenced (Secugen S.L., Madrid, Spain).

Alignments and Trees

Processing of the sequenced fragments was done using the sequence viewer and alignment editor BioEdit [43]. The fragments were reduced to a consensus sequence and trimmed to a coding sequence. These sequences combined with database sequences were aligned on the basis of their amino acid sequences and retranslated using ClustalW implemented in BioEdit [43]. As well as an input alignment, we produced an input tree to calculate the sequence evolution of *Prm1* and both domains of *Prm2* for Cricetidae. *Mus musculus* was used as the outgroup. The phylogenetic tree was built based on information gathered from the literature [31, 40, 41, 44–46] (Supplemental Fig. S1).

Evolutionary Rates (Root-to-Tip dN/dS)

The dN/dS is an indicator of selective pressure at the protein level, with dN/dS = 1 indicating neutral evolution, dN/dS < 1 purifying selection, and dN/dS > 1 diversifying positive selection [47]. To estimate the rates of sequence evolution, we used the application Codeml implemented in PAML 4 [48, 49] through the ETE toolkit [50]. The dN/dS value was generated based on the input tree and input alignment. To obtain species-specific dN/dS values to analyze the relationship between evolutionary rate and sperm head size for each species, we used the free branch model (PAML 4's Codeml) and calculated an dN/dS value for each species by addition of dN values and dS values from the root to the terminal species branch and taking the ratio of the sum to obtain the root-to-tip dN/dS value.

The association between morphometric and genetic data demands the calculation of evolutionary rates that take into account not only selective pressure acting on the terminal branch (i.e., classic free branch model, Codeml in PAML4), but also the accumulated selective pressure on the sequence during its evolution to the tip of the branch (root-to-tip dN/dS) in the selected group of taxa. Calculating an evolutionary rate in this way, values obtained become more comparable with measured phenotypical data because the latter also represent the accumulated evolution rather than being the result of changes solely on the terminal branch [24, 37].

Relative Testes Mass and Sperm Measurements

Animals were killed by cervical dislocation, weighed, and dissected immediately to remove and weigh both testes. Relative testes mass was calculated based on the rodent power function described previously [51] and used as in our previous study [24]. Mature sperm were collected from both epididymides and vasa deferentia as described [41] and suspended in a Hepes-buffered modified Tyrode medium under air [52]. Sperm dimensions were measured in sperm smears stained first with eosin-nigrosin and subsequently with Giemsa as described previously [41]. Spermatozoa were examined at 1000× under bright field, and 200 sperm cells per male were measured using ImageJ software (National Institutes of Health, Bethesda, MD).

PGLS Analysis

Associations between genetic and morphometric traits should also take into account that such traits are not independent from their phylogenetic history. The PGLS approach [53] has been shown to be a powerful tool to detect associations of this kind [54], and it has been used in earlier studies in combination with the root-to tip dN/dS method showing genetic-morphometric associations [24, 37–39]. We performed the PGLS analyses using the program COMPARE 4.6b [55]. The PGLS in COMPARE uses a single parameter, alpha (α), which can be interpreted as the fit of the comparative data with a specific evolutionary model. When $\alpha = 0$, phenotypic change (i.e., change in signal parameters) and phylogenetic distance are linearly related and, thus, the phylogenetic effect is large. When $\alpha > 0$, then phenotypic change and phylogenetic distance are exponentially related, and phylogenetic effects on trait evolution are unimportant, that is, the phylogenetic effect is very low. PGLS trait regressions using a large α are identical to standard, non-phylogenetically corrected regressions [55].

RESULTS

Sperm Measurements and Relative Testes Mass

Sperm head dimensions were analyzed in 12 species of the family Cricetidae. Measurements showed that in these species head length (HL) ranged from 4.75 μm to 8.59 μm (mean ± SEM = 6.94 ± 1.06 μm), head width (HW) varied from 2.86 μm to 4.66 μm (mean ± SEM = 3.44 ± 0.58 μm), and head elongation (HL/HW ratio) ranged from 1.54 to 2.99 (mean ± SEM = 2.06 ± 0.43). Total sperm length was also measured, and it ranged from 62.69 μm to 189.26 μm (mean ± SEM = 111.30 ± 34.01 μm). Because total sperm length varies greatly among these species, and drag resulting from head size should be analyzed taking into account the length of the flagellum [17], relative HL and HW were each calculated as percentages of total sperm length (hereafter, relative HL and relative HW). Calculated values ranged from 4.53% to 8.02% (mean ± SEM = 6.52% ± 0.34%) for relative HL and 1.51% to 4.94% (mean ± SEM = 3.37% ± 0.32%) for relative HW. Relative testes mass, which is used as a proxy of sperm competition levels, varied from 0.18 to 3.61 (mean ± SEM = 1.30 ± 1.10).

Relative Testes Mass and Sperm Head Size

To examine possible links between sperm competition on sperm head size in this dataset, we tested for correlations between species relative testes mass and sperm head measurements. We corrected for phylogenetic effects using the PGLS tool of the program COMPARE 4.6b and a phylogenetic tree reconstructed based on information gathered from the literature (Supplemental Fig. S1). The relative testes mass of cricetid species showed significant negative relationships with relative HL ($\alpha = 15.5$, CI [confidence interval] 95% (PGLS slope) = −1.02 to −0.12, correlation = −0.62) and relative HW ($\alpha = 15.5$, CI 95% (PGLS slope) = −1.10 to −0.23, correlation = −0.68) (Fig. 1, A and B, and Table 1). In addition, it showed a significant positive relationship with head elongation (HL/HW) ($\alpha = 15.5$, CI 95% (PGLS slope) = 0.78 to 2.98, correlation = 0.72) (Fig. 1C and Table 1). There were no significant correlations between relative testes mass and uncorrected HL and HW (Supplemental Table S2).

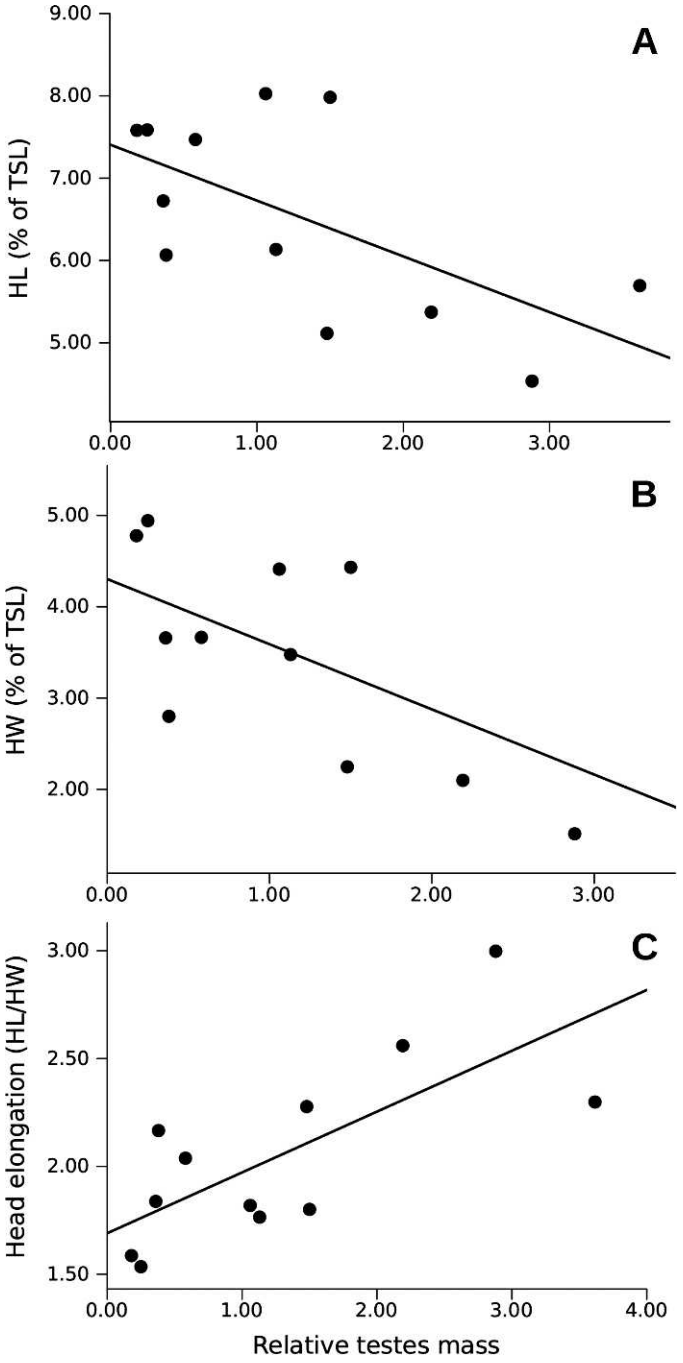

FIG. 1. Scatterplots of significant PGLS relationships between head measurements and relative testes mass. Scatterplots shown for relationships between relative sperm head length (HL) and relative testes mass (**A**), relative sperm head width (HW) and relative testes mass (**B**), and head elongation and relative testes mass (**C**). TSL, total sperm length. Dots represent species and regression lines represent least square slopes without phylogenetic correction. **A**) Slope = −0.68, $R^2 = 0.39$. **B**) Slope = −0.71, $R^2 = 0.48$. **C**) Slope = 0.28, $R^2 = 0.53$.

Evolutionary Rate of Divergence (Root-to-Tip dN/dS) and Sperm Head Size

Root-to-tip dN/dS values of branch analysis (Codeml in PAML4) ranged from 0.12 to 1.17 (mean = 0.58 ± 0.30) for *Prm1*, from 0.19 to 0.51 (mean = 0.33 ± 0.096) for cleaved-*Prm2* and from 0.28 to 1.66 (mean = 1.00 ± 0.55) for mature-*Prm2*. To test the relationship of protamine evolution with sperm head measurements, we correlated the species dN/dS value calculated from the root with the species sperm head measurements. We corrected for phylogenetic effects using the PGLS tool of the program COMPARE 4.6b.

TABLE 1. Relationships of cricetid relative testes mass with head size.

PGLS estimates	Sperm HL (%)[a]	Sperm HW (%)[a]	HL/HW[b]
CI−[c]	**−1.02**	**−1.10**	**0.78**
CI+[c]	**−0.12**	**−0.23**	**2.98**
lnL[d]	−3.82	−2.87	−2.22
Alpha[e]	15.50	15.50	15.50
Corr[f]	−0.62	−0.68	0.72

[a] Presented as percentage of total sperm length.
[b] The ratio between sperm HL and HW, which is an indicator of sperm head elongation.
[c] Confidence intervals for the PGLS regression slope (bold CI values indicate statistical significance) after PGLS analyses.
[d] The maximum likelihood estimate of alpha.
[e] The measure of evolutionary constraints acting on phenotypes; a value of 15.5 is the maximum value calculated by the program (COMPARE 4.6b).
[f] The correlation coefficient.

Prm1 root-to-tip dN/dS values for the different species showed significant positive relationships with both relative HL ($\alpha = 8.82$, CI 95% (PGLS slope) = 0.16 to 0.29, correlation = 0.90) and relative HW ($\alpha = 8.59$, CI 95% (PGLS slope) = 0.16 to 0.31, correlation = 0.88) (Fig. 2, A and B, and Table 2). On the other hand, *Prm1* root-to-tip dN/dS values showed a significant negative relationship with head elongation (HL/HW) ($\alpha = 2.78$, CI 95% (PGLS slope) = −0.73 to −0.14, correlation = −0.67) (Fig. 2C and Table 2). There was a significant relationship between the evolutionary rate of *Prm1* and uncorrected HW, but not with uncorrected HL (Supplemental Table S3).

Cleaved-*Prm2* root-to-tip dN/dS values showed significant positive relationships with relative HL ($\alpha = 7.19$, CI 95% (PGLS slope) = 0.03 to 0.10, correlation = 0.77) and with relative HW ($\alpha = 7.64$, CI 95% (PGLS slope) = 0.03 to 0.11, correlation = 0.76) (Fig. 2, D and E, and Table 2). Furthermore, cleaved-*Prm2* root-to-tip dN/dS values had a significant negative relationship with head elongation (HL/HW) ($\alpha = 7.99$, CI 95% (PGLS slope) = −0.28 to −0.08, correlation = −0.74) (Fig. 2F and Table 2). In contrast, mature-*Prm2* exhibited no significant relationship between root-to-tip dN/dS values and head measurements (Table 2). Relationships with uncorrected HL and HW were nonsignificant for cleaved-*Prm2* and mature-*Prm2* (Supplemental Table S3).

DISCUSSION

In this study, we examined genetic-morphometric associations by analyzing differences in protamine gene sequences and sperm head size and elongation with the aim of understanding the role of sperm competition on reproductive trait evolution. Earlier studies have successfully used this approach to assess genotype-phenotype associations in brain size and face length [37–39] and, to the best of our knowledge, this is the first study to explore this association in germ cells. We found significant associations between the evolutionary rates (root-to-tip dN/dS values) of *Prm1* and cleaved-*Prm2* gene sequences and sperm head morphometry, while no significant relation was found for the mature domain of *Prm2*. The evolutionary rates of both *Prm1* and the cleaved domain of *Prm2* show positive relations with relative HL and relative HW, and negative relations with head elongation (i.e., HL/HW ratio). These parameters of sperm morphometry are also

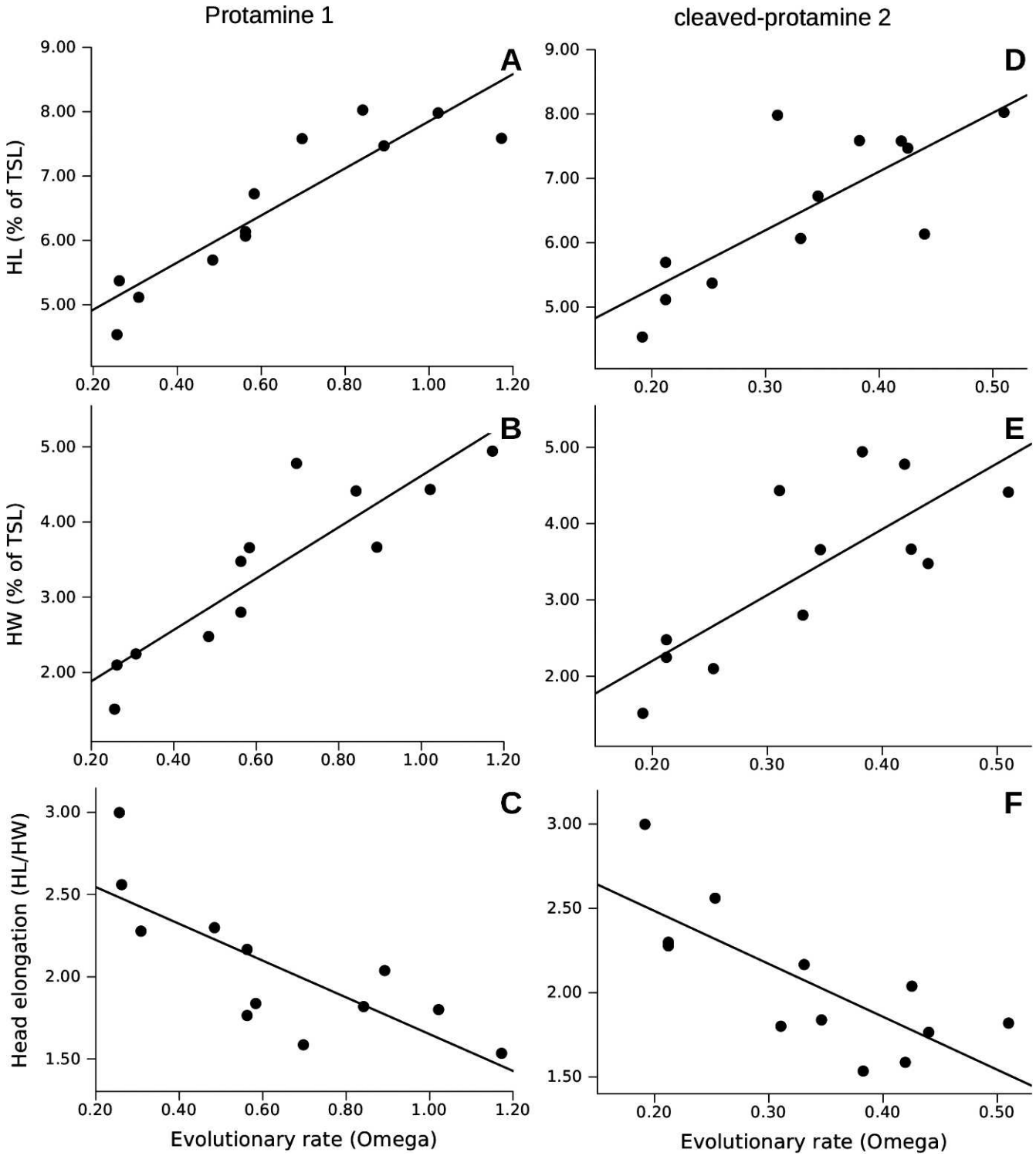

FIG. 2. Scatterplots of significant PGLS relationships between head measurements and protamine evolutionary rates (root-to-tip dN/dS). Scatterplots shown for relationships between relative sperm head length (HL) and evolutionary rate of *Prm1* (**A**), relative sperm head width (HW) and evolutionary rate of *Prm1* (**B**), head elongation and evolutionary rate of *Prm1* (**C**), relative sperm HL and evolutionary rate of cleaved-*Prm2* (**D**), relative sperm HW and evolutionary rate of cleaved-*Prm2* (**E**), and head elongation and evolutionary rate of cleaved-*Prm2* (**F**). TSL, total sperm length. Dots represent species and regression lines represent least square slopes without phylogenetic correction. **A**) Slope = 3.65, $R^2 = 0.82$. **B**) Slope = 3.41, $R^2 = 0.79$. **C**) Slope = −1.12, $R^2 = 0.60$. **D**) Slope = 9.12, $R^2 = 0.61$. **E**) Slope = 8.62, $R^2 = 0.61$. **F**) Slope = −3.14, $R^2 = 0.57$.

significantly associated with the species relative testes mass, which represent inferred levels of sperm competition [10]. Thus, these results report for the first time evidence of a relationship between differences in protamine coding sequences and sperm head shaping.

An analysis of protamine promoter sequences in mouse species found that divergence of *Prm2* promoters, which may result in differences in protamine expression, is positively correlated with sperm velocity [31]. Such a study suggested a possible effect of protamines on head shape and, hence, on the

TABLE 2. Relationships of protamine evolutionary rates with head size.

PGLS estimates	Sperm HL (%)[a]	Sperm HW (%)[a]	HL/HW[b]
Prm1			
CI−[c]	**0.16**	**0.16**	**−0.73**
CI+[c]	**0.29**	**0.31**	**−0.14**
lnL[d]	19.44	18.54	15.03
Alpha[e]	8.82	8.59	2.78
Corr[f]	0.90	0.88	−0.67
Cleaved-*Prm2*			
CI−[c]	**0.03**	**0.03**	**−0.28**
CI+[c]	**0.10**	**0.11**	**−0.08**
lnL[d]	27.48	27.40	26.88
Alpha[e]	7.19	7.64	7.99
Corr[f]	0.77	0.76	−0.74
Mature-*Prm2*			
CI−[c]	−0.02	−0.06	−0.70
CI+[c]	0.41	0.39	0.43
lnL[d]	8.19	7.82	7.11
Alpha[e]	1.32	1.07	0.59
Corr[f]	0.49	0.40	−0.15

[a] Presented as percentage of total sperm length.
[b] The ratio between sperm HL and HW, which is an indicator of sperm head elongation.
[c] Confidence intervals for the PGLS regression slope (bold CI values indicate statistical significance) after PGLS analyses.
[d] The maximum likelihood estimate of alpha.
[e] The measure of evolutionary constraints acting on phenotypes (COMPARE 4.6b).
[f] The correlation coefficient.

sperm hydrodynamic efficiency that, in consequence, may influence sperm velocity. A direct relationship between sequence divergence and sperm head shape or size in this group of closely related mouse species was not apparent [30] perhaps because subtle changes in sperm head phenotype could not be identified with the methods employed in that study.

We found positive relationships between *Prm1* and cleaved-*Prm2* divergence and relative HL and HW in cricetid rodents, a group that started diverging 16–18 million years ago [40], in contrast to a more recent emergence of mouse species, which is thought to have started about 5 million years ago [56]. A higher sequence divergence for both *Prm1* and cleaved-*Prm2* correlates with longer and wider and, therefore, bigger heads in relation to total sperm length. The relationships of sperm head elongation (HL/HW) with sequence divergence were negative for both *Prm1* and cleaved-*Prm2*. A lower sequence divergence coincided with more elongated heads. Analyses of possible associations between sperm head size and elongation with relative testes mass, which we used to infer the levels of sperm competition, revealed a different association between these parameters. Species exhibiting high relative testes mass (i.e., higher inferred sperm competition levels) showed smaller, more elongated sperm heads relative to total sperm length. This agrees with earlier comparative studies in which it was found that more elongated and smaller heads in relation to flagellum length are favored by sperm competition in mammals [13].

Elongation of the sperm head as well as lower relative sperm head size are thought to reduce drag on the sperm cell, increasing its hydrodynamic efficiency and therefore its swimming speed [17, 57]. Because swimming speed is a major factor in fertilization success [58, 59] an improvement of the hydrodynamic efficiency should be strongly favored by postcopulatory sexual selection. The correlations of evolutionary rates of *Prm1* and cleaved-*Prm2* with sperm head size and elongation, and the significant role played by head size and elongation in making sperm more competitive, support the hypothesis that protamines may influence head shape and sperm's hydrodynamic properties and sperm competitive capacity in general.

It is noteworthy that we did not find significant correlations between sequence divergence of mature-*Prm2* and sperm head size and elongation although there were strong relationships between cleaved-*Prm2* and sperm phenotype. Both PRM1 and mature-PRM2 appear to share the role of condensing DNA, whereas the function of cleaved-PRM2 is largely unknown. The uncleaved PRM2 precursor binds to DNA and is cleaved over a period of several days until only the mature-PRM2 is left bound to the DNA [60, 61]. Because DNA condensation has been found to coincide temporally with the start of protamine translation and posttranslational processing [62–64], it is reasonable to envisage that the cleaved-PRM2 domain may have a more important function during the actual process of DNA condensation than the mature-PRM2 domain. Previous studies have focused mainly on mature-PRM2, but our results suggest a more important role for cleaved-PRM2 in the process of DNA condensation and head shaping, thus warranting further studies of the role of cleaved-PRM2 and the evolution of this domain.

Our previous work on the same group of cricetid species found that both cleaved-*Prm2* and mature-*Prm2* are affected by relaxation [24]. Sexual selection was found to halt the relaxation of the *Prm2* gene, as shown by a negative relationship between sequence divergence and relative testes mass. Furthermore, *Prm1* was found to be functionally conserved with directed positive selection on specific functional sites, but it was not influenced by sexual selection [24]. In the present study, we observed that more divergent coding sequences of *Prm1* and cleaved-*Prm2* were associated with proportionately bigger and less elongated sperm heads, traits that may be less favorable in competitive situations, while higher levels of sperm competition associated with relatively smaller and more elongated sperm heads. Our current results thus suggest possible reasons why sexual selection may act to halt relaxation in *Prm2*. Higher sequence divergence in cleaved-*Prm2* seems to be related to an enlargement and reduction of elongation of the sperm head. Thus, divergence in cleaved-*Prm2* might have a negative effect on the hydrodynamic efficiency of the sperm cell, and therefore sexual selection appears to halt relaxation in *Prm2* to preclude such a decrease in hydrodynamic efficiency. Previous results showing no evidence of sexual selection on *Prm1* [24] seem to be at odds with our current results linking changes in head size and elongation with increased *Prm1* sequence divergence. However, in our previous study, *Prm1* was shown to be functionally conserved, allowing rapid changes in very specific functional sites. This functional conservation may be sufficient to ensure a hydrodynamically efficient sperm head shape in competitive situations. Because of its function and efficiency in DNA condensation, *Prm1* can be expected to affect sperm head size and elongation even though it does not appear to be influenced by sexual selection.

In conclusion, this study presents the first evidence for a potential link between divergence in protamine coding sequences and sperm head size and elongation. Because a strong correlation between sperm head phenotype and the PRM2 domain that is cleaved off during sperm chromatin condensation was found, it could be argued that this cleaved-PRM2 domain may have a role in regulating the process of DNA condensation. On the other hand, the role of mature-PRM2 (i.e., the PRM2 domain remaining after cleavage and which stays attached to DNA) might be restricted to maintenance of the condensed state of DNA in the differen-

tiated mature sperm. Lower sequence divergence in *Prm1* and cleaved-*Prm2* may be important for proportionately smaller and more elongated sperm heads, which seems to be favored by sperm competition in this group of rodents. This evidence supports previously hypothesized involvement of protamines in sperm head shaping [31]. Additionally the proposed negative influence of cleaved-*Prm2* divergence on sperm head shaping offers a possible explanation for sexual selection acting to halt relaxation of this sequence in rodents.

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REFERENCES

1. Parker GA. Sperm competition and its evolutionary consequences in the insects. Biol Rev 1970; 45:525–567.
2. Birkhead TR, Møller AP. Sperm Competition and Sexual Selection. London: Academic Press; 1998.
3. Simmons LW. Sperm Competition and Its Evolutionary Consequences in the Insects. Princeton: Princeton University Press; 2001.
4. Birkhead TR, Hosken DJ, Pitnick S. Sperm Biology: An Evolutionary Perspective. Oxford: Academic Press; 2009.
5. Swanson WJ, Vacquier, VD. The rapid evolution of reproductive proteins. Nat Rev Genet 2002; 3:137–144.
6. Turner LM, Hoekstra HE. Causes and consequences of the evolution of reproductive proteins. Int J Dev Biol 2008; 52:769–780.
7. Suarez SS, Drost M, Redfern K, Gottlieb W. Sperm motility in the oviduct. In: Bavister BD, Cummins J, Roldan ERS (eds.), Fertilization in Mammals. Norwell, MA: Serono Symposia; 1990:111–124.
8. Parker GA, Pizzari T. Sperm competition and ejaculate economics. Biol Rev 2010; 85:897–934.
9. Gomendio M, Harcourt H, Roldan ERS. Sperm competition in mammals. In: Birkhead TR, Møller AP (eds.), Sperm Competition and Sexual Selection. London: Academic Press; 1998:667–751.
10. Soulsbury CD. Genetic patterns of paternity and testes size in mammals. PLoS One 2010; 5:e9581.
11. Gomendio M, Roldan ERS. Sperm competition influences sperm size in mammals. Proc Roy Soc B 1991; 243:181–185.
12. Immler S, Moore HD, Breed WG, Birkhead TR. By hook or by crook? Morphometry, competition and cooperation in rodent sperm. PLoS One 2007; 2:e170.
13. Tourmente M, Gomendio M, Roldan ERS. Sperm competition and the evolution of sperm design in mammals. BMC Evol Biol 2011; 11:12.
14. Tourmente M, Gomendio M, Roldan ERS. Mass-specific metabolic rate and sperm competition determine sperm size in marsupial mammals. PLoS One 2011; 6:e21244.
15. Gomendio M, Roldan ERS. Implications of diversity in sperm size and function for sperm competition and fertility. Int J Dev Biol 2008; 52: 439–447.
16. Roldan ERS, Gomendio M, Vitullo AD. The evolution of eutherian spermatozoa and underlying selective forces: female selection and sperm competition. Biol Rev 1992; 67:551–593.
17. Humphries S, Evans JP, Simmons LW. Sperm competition: linking form to function. BMC Evol Biol 2008; 8:319.
18. Gómez Montoto L, Varea Sánchez M, Tourmente M, Martín-Coello J, Luque-Larena JJ, Gomendio M, Roldan ERS. Sperm competition differentially affects swimming velocity and size of spermatozoa from closely related muroid rodents: head first. Reproduction 2011; 142: 819–830.
19. Dorus S, Evans PD, Wyckoff GJ, Choi SS, Lahn BT. Rate of molecular evolution of the seminal protein gene SEMG2 correlates with levels of female promiscuity. Nat Genet 2004; 36:1326–1329.
20. Ramm SA, Oliver PL, Ponting CP, Stockley P, Emes RD. Sexual selection and the adaptive evolution of mammalian ejaculate proteins. Mol Biol Evol 2008; 25:207–219.
21. Finn S, Civetta A. Sexual selection and the molecular evolution of ADAM proteins. J Mol Evol 2010; 71:231–240.
22. Herlyn H, Zischler H. Sequence evolution of the sperm ligand zonadhesin correlates negatively with body weight dimorphism in primates. Evolution 2007; 61:289–298.
23. Walters JR, Harrison RG. Decoupling of rapid and adaptive evolution among seminal fluid proteins in Heliconius butterflies with divergent mating systems. Evolution 2011; 65:2855–2871.
24. Lüke L, Vicens A, Serra F, Luque-Larena JJ, Dopazo H, Roldan ERS, Gomendio M. Sexual selection halts the relaxation of protamine 2 among rodents. PLoS One 2011; 6:e29247.
25. Oliva R, Dixon GH. Vertebrate protamine genes and the histone-to-protamine replacement reaction. Prog Nucleic Acid Res Mol Biol 1991; 40:25–94.
26. Oliva R. Protamines and male infertility. Hum Reprod Update 2006; 12: 417–435.
27. Balhorn R. The protamine family of sperm nuclear proteins. Genome Biol 2007; 8:227.
28. Krawetz SA, Dixon GH. Sequence similarities of the protamine genes, implications for regulation and evolution. J Mol Evol 1988; 27:291–297.
29. Wyckoff GJ, Wang W, Wu WJ. Rapid evolution of male reproductive genes in the descent of man. Nature 2000; 403:304–309.
30. Rooney AP, Zhang J. Rapid evolution of a primate sperm protein, relaxation of functional constraint or positive Darwinian selection? Mol Biol Evol 1999; 16:706–710.
31. Martin-Coello J, Dopazo H, Arbiza L, Ausió J, Roldan ERS, Gomendio M. Sexual selection drives weak positive selection in protamine genes and high promoter divergence, enhancing sperm competitiveness. Proc Biol Sci 2009; 276:2427–2436.
32. Cho C, Jung-Ha H, William WD, Goulding EH, Stein P, Xu Z, Schultz RM, Hecht NB, Eddy EM. Protamine 2 deficiency leads to sperm DNA damage and embryo death in mice. Biol Reprod 2003; 69:211–217.
33. Aoki VW, Liu L, Carrell DT. Identification and evaluation of a novel sperm protamine abnormality in a population of infertile males. Hum Reprod 2005; 20:1298–1306.
34. Carrell DT, Emery BR, Hammoud S. Altered protamine expression and diminished spermatogenesis: what is the link? Hum Reprod Update 2007; 13:313–327.
35. Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi Y, Hecht NB, Eddy EM. Haploinsufficiency of protamine-1 or −2 causes infertility in mice. Nat Genet 2001; 28:82–86.
36. Belokopytova IA, Kostyleva EI, Tomilin AN, Vorob'ev VI. Human male infertility may be due to a decrease of the protamine P2 content in sperm. Mol Reprod Dev 1993; 34:53–57.
37. Montgomery SH, Capellini I, Venditti C, Barton RA, Mundy NI. Adaptive evolution of four microcephaly genes and the evolution of brain size in anthropoid primates. Mol Biol Evol 2011; 28:625–638.
38. Pointer MA, Mamilar JM, Warmuth V, Chester SGB, Delsuc F, Mundy NI, Asher RJ, Bradley BJ. RUNX2 tandem repeats and the evolution of facial length in placental mammals. BMC Evol Biol 2012; 12:103.
39. Montgomery SH, Mundy NI. Positive selection on NIN, a gene involved in neurogenesis, and primate brain evolution. Genes Brain Behav 2012; 11:903–910.
40. Steppan SJ, Adkins RM, Anderson J. Phylogeny and divergence-date estimates of rapid radiations in muroid rodents based on multiple nuclear genes. Syst Biol 2004; 53:533–553.
41. Gómez Montoto L, Magaña C, Tourmente M, Martín-Coello J, Crespo C, Luque-Larena JJ, Gomendio M, Roldan ERS. Sperm competition, sperm numbers and sperm quality in muroid rodents. PLoS One 2011; 6:e18173.
42. Corzett M, Kramer C, Blacher R, Mazrimas J, Balhorn R. Analysis of hamster protamines, primary sequence and species distribution. Mol Reprod Dev 1999; 54:273–282.
43. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids S 1999; 41:95–98.
44. Jaarola M, Martínková N, Gündüz I, Brunhoff C, Zima J, Nadachowski A, Amori G, Bulatova NS, Chondropoulos B, Fraguedakis-Tsolis S, González-Esteban J, López-Fuster MJ, et al. Molecular phylogeny of the speciose vole genus *Microtus* (Arvicolinae, Rodentia) inferred from mitochondrial DNA sequences. Mol Phylogenet Evol 2004; 33:647–663.
45. Galewski T, Tilak M, Sanchez S, Chevret P, Paradis E, Douzery EJ. The evolutionary radiation of Arvicolinae rodents (voles and lemmings), relative contribution of nuclear and mitochondrial DNA phylogenies. BMC Evol Biol 2006; 6:80.
46. Neumann K, Michaux J, Lebedev V, Yigit N, Colak E, Ivanova N, Poltoraus A, Surov A, Markov G, Maak S, Neumann S, Gattermann R. Molecular phylogeny of the Cricetinae subfamily based on the mitochondrial cytochrome b and 12S rRNA genes and the nuclear vWF gene. Mol Phylogenet Evol 2006; 39:135–148.
47. Goldman N, Yang Z. A codon-based model of nucleotide substitution for protein-coding DNA sequences. Mol Biol Evol 1994; 11:725–736.
48. Yang Z, Rannala B. Bayesian phylogenetic inference using DNA sequences, Markov chain Monte Carlo methods. Mol Biol Evol 1997; 14:717–724.

49. Yang Z. PAML 4, phylogenetic analysis by maximum likelihood. Mol Biol Evol 2007; 24:1586–1591.
50. Huerta-Cepas J, Dopazo J, Gabaldon T. ETE: a python environment for tree exploration. BMC Bioinformatics 2010; 11:24.
51. Kenagy GJ, Trombulak SC. Size of mammalian testes in relation to body size. J Mammal 1986; 67:1–22.
52. Shi QX, Roldan ERS. Bicarbonate/CO_2 is not required for zona-pellucida-induced or progesterone-induced acrosomal exocytosis of mouse spermatozoa but is essential for capacitation. Biol Reprod 1995; 52:540–546.
53. Pagel, M. The maximum likelihood approach to reconstructing ancestral character states of discrete characters on phylogenies. Syst Biol 1999; 48: 612–622.
54. Rholf FJ. Comparative methods for the analysis of continuous variables: geometric interpretations. Evolution 2001; 55:2143–2160.
55. Martins EP. COMPARE 4.6b: phylogenetic comparative methods. Bloomington, IN: Indiana University, Department of Biology, http://compare.bio.indiana.edu/. Accessed 9 April 2012.
56. Guénet JL, Bonhomme F. Wild mice: an ever-increasing contribution to a popular mammalian model. Trends Genet 2003; 19:24–31.
57. Malo AF, Gomendio M, Garde J, Lang-Lenton B, Soler AJ, Roldan ERS. Sperm design and sperm function. Biol Lett 2006; 2:246–249.
58. Birkhead TR, Martinez JG, Burke T, Froman DP. Sperm mobility determines the outcome of sperm competition in the domestic fowl. Proc Roy Soc Lond B 1999; 266:1759–1764.
59. Gage MJG, Macfarlane CP, Yeates S, Ward RG, Searle JB, Parker GA. Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. Curr Biol 2004; 14:44–47.
60. Chauvière M, Martinage A, Debarle M, Sautière P, Chevaillier P. Molecular characterization of six intermediate proteins in the processing of mouse protamine P2 precursor. Eur J Biochem 1992; 204:759–765.
61. Carre-Eusebe D, Lederer F, Le KH, Elsevier SM. Processing of the precursor of protamine P2 in mouse. Peptide mapping and N-terminal sequence analysis of intermediates. Biochem J 1991; 277:39–45.
62. Lee K, Haugen HS, Clegg CH, Braun RE. Premature translation of protamine 1 mRNA causes precocious nuclear condensation and arrests spermatid differentiation in mice. Proc Natl Acad Sci U S A 1995; 92: 12451–12455.
63. Brewer L, Corzett M, Balhorn R. Condensation of DNA by spermatid basic nuclear proteins. J Biol Chem 2002; 277:38895–38900.
64. Kierzenbaum AL, Tres LL. Structural and transcriptional features of the mouse spermatid chromosome. J Cell Biol 1975; 65:258–270.

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Lena Lüke, Polly Campbell, María Varea Sánchez, Michael W. Nachman and Eduardo R. S. Roldan

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